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MEDICAL SECTION OF NATIONAL TUBERCULOSIS ASSOCIATION

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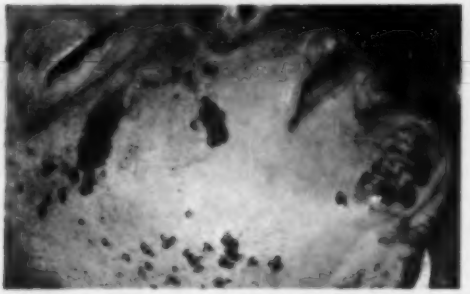

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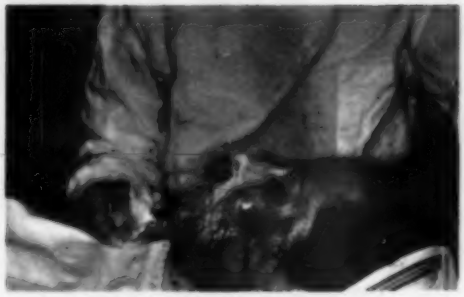

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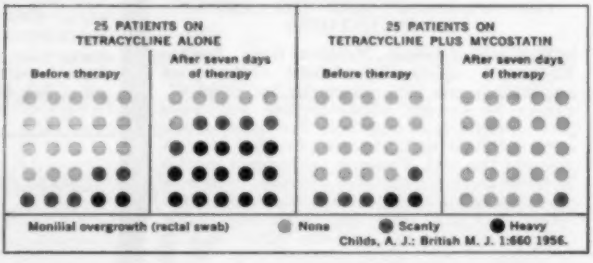

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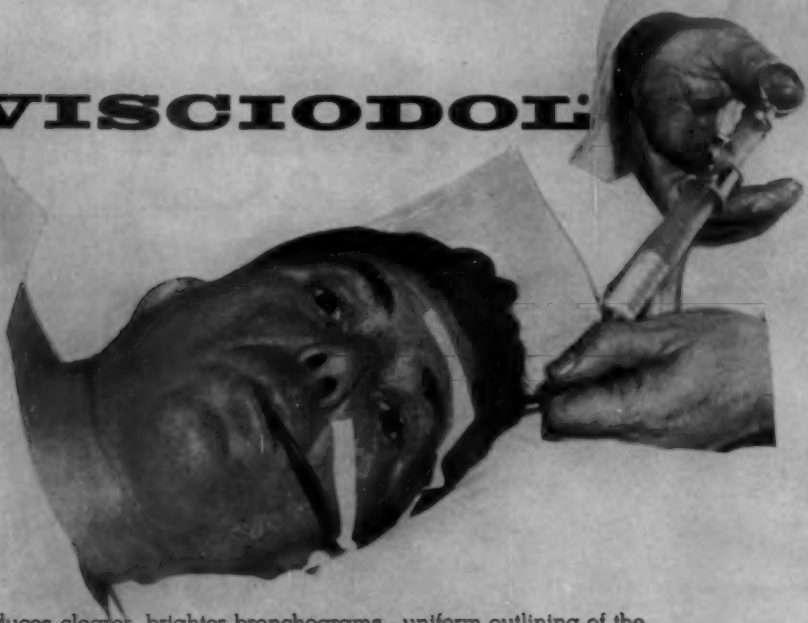

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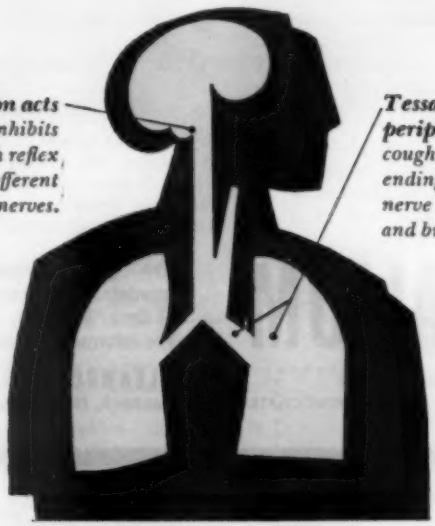
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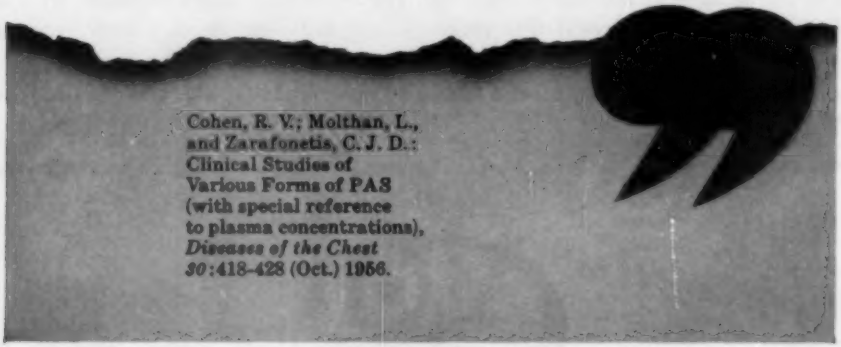

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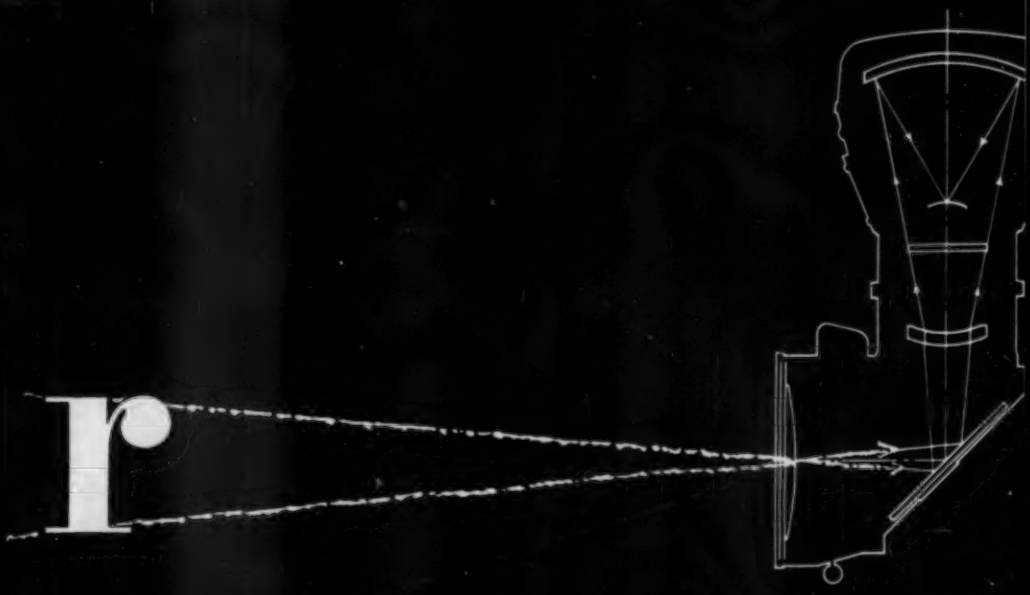

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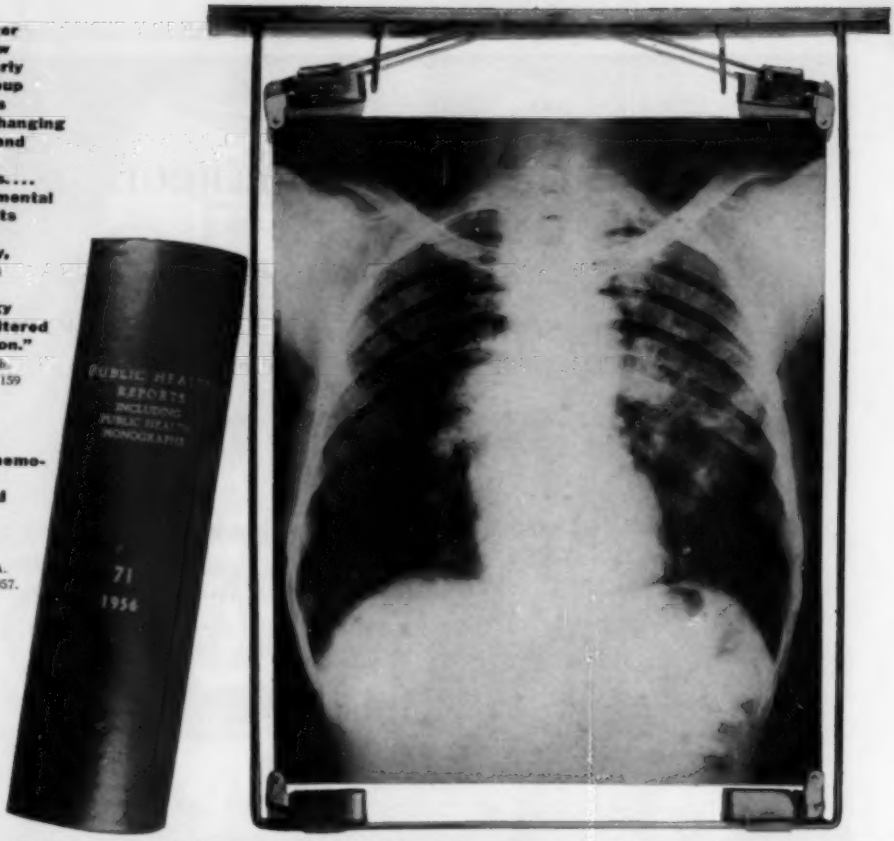

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1958 ANNUAL MEETING

PHILADELPHIA, PENNSYLVANIA, MAY 18–23

The 1958 Annual Meetings of the National Tuberculosis Association, the American Trudeau Society, and the National Conference of Tuberculosis Workers will be held at Convention Hall, Philadelphia, Pa., May 18 to 23.

The ATS committee meetings will be held Friday, Saturday, and Sunday, May 16, 17, and 18.

Registration will begin Saturday afternoon, May 17, and continue through Sunday evening at the Bellevue-Stratford Hotel. Beginning Monday, May 19, registration, the exhibits program, and regular daytime sessions will be held at Convention Hall. Evening sessions through the week will be held at the Bellevue-Stratford.

Medical, public health, and nursing sessions will begin on Monday, May 19. General sessions will include an opening keynote program Monday A.M., the awards session Wednesday evening, and a session Thursday morning, May 22.

Blocks of rooms have been reserved in the following hotels.

Bellevue-Stratford. Rates: single, $9–11; double, $15; twins, $12—17.
Adelphia. Rates: single, $7—10; double, $12—14; twins, $13—16.
John Bartram. Rates: single, $7; double, $9.50—10.50; twins, $10.50—12.50.
Sheraton. Rates: single, $9.85—12; twins, $14—17.
Sylvania. Rates: single, $7—7.50; twins, $11.50—13.50.
Warwick. Rates: single, $11; double, $13—16; twins, $14—19.

Special bus service will be provided between these hotels and Convention Hall.

Applications for rooms should be made directly to the hotel of choice. *Requests must include mention of NTA Annual Meeting and give a definite date and hour of arrival as well as a date of departure. Names and addresses of all persons who will occupy the rooms requested must be included.* In the event the hotel is unable to provide the desired space, requests will be referred to the Philadelphia Convention Bureau for clearing with another one of the cooperating hotels. Confirmation will come direct from hotel accepting reservation.

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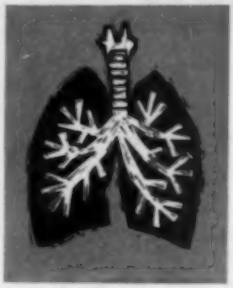

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Allergic asthma 2	2-10	2		
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1. Reports to the Squibb Institute of Medical Research, 1957. 2. Sherwood, H., and Cooke, R. A.: J. Allergy 28:97, 1957. 3. Hellman, L., et al.: American Rheumatism Association. Interim session, Nov. 23, 1956. 4. Berntsen, C. A., Jr., et al.: New York Rheumatism Association. Annual meeting, April 9, 1957, New York. 5. Freyberg, R. H., et al.: International Congress on Rheumatic Diseases, June, 1957, Toronto.

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THE AMERICAN REVIEW OF TUBERCULOSIS AND PULMONARY DISEASES

VOLUME 77 *March, 1958* NUMBER 3

THE HAZARDS OF RADIATION[1,2]

DAVID M. GOULD AND WALTER MAUDERLI

(Received for publication October 24, 1957)

INTRODUCTION

Early in this century the people who worked with X-ray equipment and radium had no conception of the danger involved. Doctors and technicians held radium in their fingers. They tested X-ray machines and fluoroscopes by examining the bones in their own hands. Many of these people died martyrs to the "Age of the Atom" which they themselves had helped to usher in. A few of these men are still living. Dr. Emil Grubbe is eighty-two years old. He lives in Chicago and has had ninety operations for skin cancer from radiation burns suffered more than fifty years ago (1).

ACTION OF RADIATION

Penetrating rays act in matter by ionization. The exact mechanism of action in living cells is not known. It is likely that the ionizing rays interfere with the enzyme systems and do particular damage to the nucleus of the cell. It is also likely that the nucleus is sensitive to ionizing rays because of the crucial nature of chromatin which forms the chromosomes which in turn are composed of genes. Chromatin, chromosomes, and genes are probably formed from a complex chemical called desoxyribonucleic acid, commonly known as DNA.

The cells most sensitive to ionizing radiation are the cells which reproduce fastest. In the body the average length of life of the cell is an indication of the rate of reproduction of the parent cell. A lymphocyte lives about three days. Thrombocytes or platelets remain intact for four to nine days. An epithelial cell of the intestinal mucosa lives about four days. A granulocyte lives about seven to eleven days. A mature erythrocyte exists for one hundred and twenty days, but since it has no nucleus it is not really a living cell and is not sensitive to radiation. However, the erythroblast, which is the parent of the erythrocyte, is sensitive to radiation. Thus, erythroblast damage will eventually result in anemia.

There are some cells which live almost the entire life of the individual. Ex-

[1] From the Department of Radiology, University of Arkansas School of Medicine, Little Rock, Arkan[illegible].

[2] Presented at th[illegible]nual meeting of the National Tuberculosis Association, Kansas City, Missouri, on Ma[illegible]3, 1957.

amples of these cells are: heart muscle fibers, striated muscle cells, and neurons. These mature cells do not reproduce and are quite insensitive to radiation.

The effects of ionizing rays resemble the aging process. Scattered cells become more variable in size, shape, and chromatin content. The older cells aggregated in the organism show evidence of useless metabolic products, such as certain pigments, and show cytoplasmic atrophy. Under the influence of radiation, products such as melanin tend to decrease; collagen tends to hyalinize; elastic tissue tends to degenerate. Glucksmann (2) stresses the increased differentation of cells after irradiation.

LOCAL SKIN IRRADIATION

The effects of radiation on the skin develop insidiously and have long been familiar. After an acute exposure there erythema develops, usually within two weeks. Months and years later there are changes in pigmentation, epilation. drying of the sweat and sebaceous glands, atrophy telangiectasis, ulceration, and hyperkeratosis. Many years later epidermoid carcinoma may develop in the skin that is severely damaged by radiation. It is paradoxical that penetrating rays have the power to both cure and produce cancer.

ACUTE WHOLE BODY IRRADIATION

In whole body irradiation the blood-forming system, the gastrointestinal tract, and the skin are most sensitive in this order. The supply of leukocytes, thrombocytes, gamma globulin, and later erythrocytes is depleted. Ulcerations develop in the gastrointestinal tract because the normal regeneration of epithelial cells cannot occur. The skin temporarily loses its hair.

In whole body irradiation with a dose of 450 roentgens, approximately 50 per cent of humans die (tables 1, 2, and 3).

A roentgen is defined as a measure of an amount of ionization which takes place when a beam or radiation passes through 1 cc. of air. When a beam, no matter how large or small, and regardless of the time involved, produces 2.083 billion ionizations per 1 cc. of air through which it passes, the radiation equals one roentgen. (For more precise definition, *see* figure 1.) If tissue is exposed to one roentgen in the region of one million volt radiation, the energy dissipated is 93 ergs. As the roentgen is in itself the unit, it is redundant to speak in terms of roentgen "units."

TABLE 1

ACUTE GAMMA-RAY OR X-RAY SYNDROME AFTER WHOLE BODY IRRADIATION
Sublethal Dose, 100–250 r

First and second weeks	No clear symptoms
Third week	Beginning epilation, loss of appetite, general malaise
Fourth week	Sore throat, malaise, petechiae, diarrhea, moderate weakness

Recovery probable, barring complications.

TABLE 2

ACUTE GAMMA-RAY OR X-RAY SYNDROME AFTER WHOLE BODY IRRADIATION
Moderately Lethal Dose, 400-600 r

First and second weeks	No clear symptoms
Third week	Beginning epilation, loss of appetite, general malaise, fever
Fourth week	Inflammation of mouth and throat, petechiae, diarrhea, nose bleed, rapidly increased weakness, death

Mortality about 50 per cent

TABLE 3

ACUTE GAMMA-RAY OR X-RAY SYNDROME AFTER WHOLE BODY IRRADIATION
Lethal Dose, 700 r or greater

1 to 2 hours after irradiation	Nausea and vomiting
First week	Diarrhea, vomiting, inflammation of mouth and throat
Second week	Fever, rapidly increasing weakness, death

Mortality nearly 100 per cent

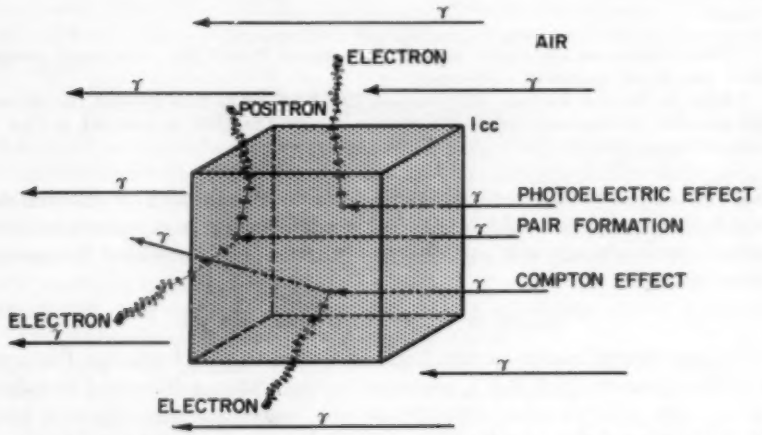

FIG. 1. Definition of the roentgen (r). The amount of X- or γ-radiation is 1r, when the secondary particles, created in 1 cc. of air at 0°C. and 760 mm. of mercury (1.293 mg.), produce ions which carry 1 electrostatic unit of electricity of either sign (about 2 billion ion pairs).

The *roentgen-equivalent-physical* (rep) is the unit of absorbed dose equal to 93 ergs per gm. of tissue. This unit is now superseded by the rad which is the unit of absorbed dose equal to 100 ergs per gm. The *roentgen-equivalent-man* (rem), more properly the *rad-equivalent-man*, is a unit of dose useful in protec-

TABLE 4

UNITS FOR RADIATION DOSIMETRY AND THEIR COMPARISON

Unit	Definition	Comparison
Roentgen (r)	1 r is such an amount of X- or gamma radiation that the associated corpuscular emission per 0.001293 gm. of air produces, in air, ions carrying 1 esu of quantity of electricity of either sign	In case of tissue irradiation: 1 r is about equal to 1 rep* or 0.93 rad*
Roentgen equivalent physical (rep)	1 rep is an absorbed dose of ionizing radiation of 93 ergs per gm. of tissue, independent of tissue composition and type of ionizing radiation.	1 rep is equal to 0.93 rad and in case of tissue irradiation about equal to 1 r*
Rad	1 rad is an absorbed dose of ionizing radiation of 100 ergs per gm. of material independent of tissue composition and type of ionizing radiation.	1 rad is equal to 1.075 rep and in case of tissue irradiation about equal to 1.08 r*
Rad equivalent man (rem)	1 rem = 1 rad × RBE†	

* These figures are essentially correct for γ-rays of 1 to 2 MeV. For lower energies, there may be an appreciable difference.

† RBE = Relative biologic effectiveness. The RBE takes into account the different biologic effect for the same absorbed radiation energy. The RBE is assumed as 1 for X-, γ- and β-rays.

tion and is equal to the relative biologic effectiveness multiplied by absorbed dose in rads. This unit is helpful in comparing or adding one type of ionizing radiation such as electron beams with any other type such as alpha particles or X or gamma radiation (table 4).

MAXIMUM PERMISSIBLE DOSE (M.P.D.)

To help control radiation, the National Committee on Radiation Protection and Measurement (N.C.R.P.), sponsored by the National Bureau of Standards, issues from time to time recommendations concerning safe exposure levels. In 1949 the maximum permissible dose (M.P.D.) recommended to persons working with radiation was set at 300 mr (0.3 r) per week. Recently the Committee recommended a lower yearly and total accumulated dose (3). It is quite possible that the weekly limit of 300 mr will be lowered to 100 mr. (table 5.)

LATE EFFECTS OF CHRONIC SMALL-DOSE IRRADIATION

In recent years scientists began to realize that chronic small doses of radiation can also damage the individual. An early clue came in 1915 from the luminous clock-dial workers in New Jersey who dipped a brush into zinc sulphide radium

TABLE 5

MAXIMAL PERMISSIBLE DOSE FOR X- AND GAMMA-RAYS UP TO 3 MeV
Occupational Conditions

Total accumulated dose	(Age minus 18 years) times 5 rems 5 (N-18)
Yearly dose	5 r
Weekly dose	0.3 r (300 mr)
Daily dose	0.05 r (50 mr)

These values apply to all critical organs except the skin, for which the value is doubled.

mixture and pointed the brush between pursed lips. Many died of radium poisoning which was accompanied by anemia, infection, necrosis of the jaw, and pathologic fractures of bone. Osteogenic sarcoma has been reported in more than 60 patients who have ingested radium. The usual latent period was twenty-five years.

From 1930 to 1945 thorium dioxide (Thorotrast®) was used to opacify the liver and spleen. This colloid is retained in the reticuloendothelial system throughout life. The alpha particles emitted from the thorium produced induration, contractures, and occasionally malignant tumors.

Simpson and Hempelman (4) studied the association of tumors with roentgen-ray treatment of the thorax in infancy. Among 1,502 children treated for thymic enlargement, there were 18 malignant tumors, 10 cases of carcinoma of the thyroid, and 8 of leukemia. Among the 1,933 untreated siblings there were 5 malignant tumors.

RADIATION-INDUCED LEUKEMIA

A study of patients who received radiation treatment for ankylosing spondylitis (Marie Strumpel's disease) showed a considerable increase in the incidence of leukemia (5).

The survivors near the atom bomb hypocenter had a twelvefold increased incidence in leukemia compared to the survivors of the periphery. The latent period was three to six years.

March (6), in a statistical analysis, found that radiologists die of leukemia about nine times more frequently than other physicians.

From these various data, Lewis (7) calculated that the risk of radiation-induced leukemia per roentgen per year was 2 persons out of a million. In other words, if an individual were occupationally exposed to 250 r (a rather large dose and equal to the total maximal accumulated permissible dose for a person 68 years of age), the probability of his developing leukemia would be one chance in 2,000 per year.

While the relative death rates would appear high, it must be remembered that the absolute number of leukemic deaths among radiologists would be low. In the analysis of March, 6 of about 4,000 radiologists had died in a five-year period. The small absolute figures made it difficult to realize the statistical relationship of cause and effect between radiation and leukemia (8). The risk of radiation-induced leukemia may be compared with more familiar risks, such as the risk of

lung cancer from smoking cigarettes or the risk of dying in an automobile accident.

If an average individual smoked at the rate of one package of cigarettes a day, the risk of developing lung cancer would be 860 chances per million per year, or a little less than one chance in 1,000 each year.[3] This yearly risk is about twice that of total body exposure to 250 r.

The risk of accidental death from a motor vehicle driven 10,000 miles per year would be 640 chances per million per year.[4] This yearly risk would be approximately the same as developing leukemia with an exposure of 250 r.

It may be concluded that smoking a package of cigarettes a day is more dangerous than driving 30 miles a day or accumulating fifty years of radiation at the rate of 5 roentgens per year.

DECREASED LONGEVITY FROM RADIATION

Warren (11) claims that radiologists die on the average of 5.2 years earlier than other physicians. The non-radiology specialists known to be exposed somewhat to radiation also seem to show shortening of life. In the case of gastroenterologists, tuberculosis specialists, and urologists, the average life span is two years less than that of physicians not exposed to radiation. The work of Warren has not been corroborated by others.

It is not sufficient to appreciate the acute lethal radiation hazard to the individual, nor is it sufficient to study the subtle radiation hazard, continuing over decades, to thousands of people who are engaged in the field of radiation. With the exploding of the atom bomb new dimensions have been added. It is now necessary to appreciate the hazard of infinitesimal doses of radiation involving all of living mankind and the unborn generations to come. The facts presented by the geneticists in considering this subject are certainly disturbing (12).

RADIATION HAZARDS TO FUTURE GENERATIONS (13, 14)

It is well recognized that genes are present in all body cells, and mutation of these genes may be produced by heat, chemical action, or radiation. In the somatic or ordinary body cells these mutations with other factors may lead to neoplastic changes, aging, or decreased longevity. When the reproductive organs or gonads are exposed to radiation, sudden destruction or change in the genes brings up mutations which may cause the offspring to differ radically from the parents. These mutations may persist in the race to the last child born unless eliminated by natural selection. Genetic death of an undesirable gene results from sterility, death of embryo or fetus, or death before reproduction. On very rare occasions a mutation may produce a superb individual; but, in most instances,

[3] "Analysis of the data relating to men has shown a marked and steady increase in the mortality from lung cancer as the amount smoked increases. Thus, at ages 35 and over, the death rate per year rose from 0.07 per 1,000 in nonsmokers—based upon the observation of one death only—to 0.47 per 1,000 in smokers of 14 grams a day; to 0.86 per 1,000 in smokers of 15 to 24 grams a day; and finally to 1.66 per 1,000 in smokers of 25 grams or more a day—one gram of tobacco is approximately the amount contained in one cigarette."(9)

[4] Death rate of motor vehicle accidents in 1956: 6.4 per 100,000,000 vehicle miles (10).

TABLE 6

NATURAL RADIATION SOURCES (15)

	Radiation Dose Per Year
1. Radioactive isotopes in the body	
Potassium 40: average amount in microcuries 0.1	20 m rad
Carbon 14: average amount in microcuries 0.06	1 m rad
Radium 226 + disintegration products 0.001	2 m rad
2. External radiation	
Cosmic rays	28 m rad
Local surrounding gamma rays	46 m rad
Total dose per year	97 m rad
Total dose to age 30 years	2.91 rad

it produces undesirable characteristics such as hereditary defects, congenital malformations, and constitutional weakness.

From experiments with plants and animals, and a study of people in Nagasaki and Hiroshima, geneticists came to the conclusion that any radiation which penetrated to the reproductive cells and the gonads was harmful to future generations. This damage is accumulative and statistically it makes no difference whether the gonads receive 100 r over a thirty-year period or whether 100 r are given in one minute. Furthermore, from a broad population viewpoint it makes no difference if 100 people receive 100 r or 1,000 people receive 10 r. The general pool of genes is affected the same way.

Society as a whole can allow certain small select groups to be exposed to higher doses than the general population. In occupations with radiation hazards, such as radiology, uranium mining, and nuclear reactor maintenance, the exposure should be kept to 50 r during the first 30 years and should not exceed another 50 r between 30 and 40 years of age. Geneticists warn society that radiation damages the stuff of inheritance and causes mutations which are generally harmful.

Natural radiation, including cosmic rays, has been present ever since the earth was created. Cosmic rays include some of the most penetrating rays known. Some cosmic rays could go through many inches of lead or many feet of earth. Cosmic rays also change certain atoms into radioactive atoms.

In our own bodies there is a certain amount of radioactive potassium, carbon, and even radium. These atoms are present, of course, in infinitesimal amounts, but they are measurable. They also irradiate us continuously (table 6).

According to the best estimates of scientists working with the National Academy of Science, people in general receive from natural irradiation about 3 r during their lives from birth to the age of thirty years. A little more might be received from cosmic rays because of the altitude in certain localities, for example, in Denver, Colorado. Granite has a relatively high natural radioactivity so that people who live in stone houses may receive a little more radiation than people living in brick houses. People living in wooden houses receive even less than those who live in brick houses. Uranium miners receive large exposures. It has

long been noted that in Schneeburg, Germany, and in Joachimsthal, Czechoslovakia, there is so much inhaled radioactive material that many workers develop cancer of the lung.

It may be assumed, therefore, that the average natural radiation from both inside and outside the body would amount to about 3 r during the first thirty years of life. The period of the first thirty years of life is used because one-half of the number of children born in this country are born from fathers less than thirty years old and mothers less than twenty-eight years old. Ninety per cent of the children born are from parents less than forty. After the age of forty, therefore, an individual's exposure to radiation is not likely to harm the inheritance of the human race.

In addition to natural radiation there is also man-made radiation. Man-made radiation results from radioisotopes and X-ray generating equipment. The atom bomb generates large quantities of isotopes. However, from the long-range point of view, the hazard from nuclear power reactors may be greater than that of the atomic bomb. "Little atom bombs" like the ones used at Nagasaki and Hiroshima are measured in thousands of tons of TNT or kilo-tons, and develop a radioactive fall-out of perhaps a few thousand square miles. This fall-out consists of many radioactive elements with varying half lives and includes strontium 90, half of which disintegrates in twenty-eight years. Metabolically and nutritionally radioactive strontium seems to affect plants and animals much like calcium. Most of the ingested radioactive strontium is deposited in the bones and remains for years.

The power of the newer hydrogen bomb is measured in megatons, or millions of tons of TNT. They explode so violently that they reach very high altitudes and their products are widely scattered about the world. The fall-out comes down by gravity, the precipitation of rain or snow, convection, and diffusion currents.

It has been estimated that with all of the bombs detonated thus far and the continuation of detonation at the present rate, individuals will absorb about 0.1 roentgen over a period of about thirty years. The children of today have more radiostrontium in their bones than did their parents when they were children.

The estimate of radiation used for medical purposes accounts for about 4 roentgens during the first thirty years of life. Some people, of course, receive considerably more while others receive none at all.

Considering all of these facts, the geneticists have estimated that mankind would not be unduly harmed if an average of 10 roentgens over and above natural radiation were absorbed by the reproductive cells during the first thirty years of life. The National Committee on Radiation Protection (N.C.R.P.) states "the maximum permissible dose (M.P.D.) to the gonads for the population of the United States as a whole from all sources of radiation, including medical and other man-made sources, and background, shall not exceed 14,000,000 rem per million population over the period from conception to the age of 30, and one-third that amount in each decade thereafter" (average 14 rem per individual).

To the 3 roentgens from natural radiation must be added approximately 4 roentgens from medical irradiation and roughly 0.1 roentgen from the irradiation

scattered by the atomic bomb. The total is thus slightly more than 7 roentgens. As the permissible limit from a genetic viewpoint is 14 roentgens including natural radiation, we are approaching today the limit of a genetically safe level. It is as though every individual is born with 14 roentgens "credit" in the bank. He is forced to spend roughly 3 roentgens on natural radiation and will probably spend more than 4 roentgens on man-made sources of radiation. Therefore, the average individual usually only has 7 roentgens "credit" in the bank, or by the time he is thirty has spent one-half of his radiation "credit."

What if society disregards these warnings and radiation accumulates due to a lack of control? When the average level of radiation for the first thirty years of life reaches between 30 and 80 roentgens, there will be a doubling of the present number of mutations. The present burden of 2 per cent of hereditarily defective children will be increased to 4 per cent.

What can be done about the radiation hazard? The ideal goal, but impossible to achieve, would be no radiation at all to the gonads from birth to the end of the reproductive period of man. However, we certainly cannot do anything about the 3 roentgens of natural radiation. We are left with the possible controllable sources—medical radiation and radiation caused by atomic bomb explosions and atomic power plants.

The possible harm from atom bomb testing must be weighed by society in the light of the need for national defense. Thus far, the atom bomb radiation would appear to be a rather small hazard. However, if the rate of testing increases greatly or if nuclear power installations are not very carefully controlled, the contamination of the whole world with radioactive materials would pose a grave hazard.

The control of medical radiation poses a moral dilemma in which the health and welfare of the individual must be weighed against the hazard to future generations. Faced with this choice, we would favor the welfare of the patient now. Self-preservation is a more powerful instinct than race preservation.

There are many ways however, by which the medical radiation hazard can be diminished and still do justice to the individual while minimizing genetic damage.

First, any X-ray generator that is not supervised by specially trained personnel should be banned. Every state should ban shoe-fitting fluoroscopy. These machines are useless except for advertising wares. The hazard is all the more serious because children are usually subjected to their rays.

EXPOSURE FROM DIAGNOSTIC EXAMINATIONS (16)

An ordinary chest film delivers 0.04 roentgens to the chest and less than 1 milliroentgen to the gonads. The regular photofluorography delivers 1 to 2 roentgens to the chest and 5 milliroentgens to the gonads. The newer mirror-optics-system photofluorograph delivers 1 roentgen to the chest and less than 1 milliroentgen to the gonads. Although it may be calculated that, if 10 roentgen gonadal radiation were to be used up entirely by conventional photofluorographic chest films, it would take more than 2,000 chest exposures during the first thirty years of life, this calculation does not take into account the hazard to the in-

TABLE 7

Doses for Routine Diagnostic X-Ray Examinations

Author	Examination	Dose		
		Skin at Beam Entrance	Gonads	
			Male	Female
Gould and Mauderli	Chest fluoroscopy: conventional type	3–10 r/min.	2–10 mr/min.	
Sorrentino and Yalow (17)		6.6–17.3 r/min. (air)		
Ritter *et al.* (18)		11.5–17.5 r/min.		
Gould	Chest fluoroscopy: Image intensifier	1–2 r/min.	less 1 mr/min.	
Gould	Photofluorography of chest: conventional type	1–2 r/film	1–5 mr/film	
Billings *et al.* (19)			1.0 mr/film	3.0 mr/film
Gould	Photofluorography of chest, new mirror optic system	0.5–1 r/film	less 1 mr/film	
Gould	Chest film, posteroanterior	15–40 mr/film	less 1 mr/film	
Billings		15 mr/film	0	0
Ritter		60 mr/film		
Sorrentino		35 mr/film (air)		
	Pelvis: Pregnancy			
Gould	Anteroposterior	2 r/film		0.3 r/film
	Lateral	3 r/film		0.4 r/film
Martin (20)	Pregnancy	10–24 r/film		0.2 r/film
Billings	Anteroposterior	0.5 r/film	0.5 r/film	0.2 r/film
Ritter	Anteroposterior	5 r/film		0.4 r/film
	Lateral	26.7 r/film		3.5 r/film
Sorrentino	Anteroposterior	1.3 r/film (air)		
	Excretory pyelogram:			
Gould	Anteroposterior,	1 r/film	10 mr/film	120 mr/film
	oblique	1.5 r/film	10 mr/film	180 mr/film
Ritter	Anteroposterior,	1.3 r/film		
	oblique	1.4 r/film		
Sorrentino	Anteroposterior,	1.2 r/film (air)		
	oblique	1.6 r/film (air)		
	Lateral	3.2 r/film (air)		
Gould	Skull	1–2 r/film	less 1 mr/film	less 1 mr/film
Ritter		2.8–7.2 r/film		
Sorrentino		1.3–1.7 r/film (air)		
Gould	Dental film	2 r/film	less 1 mr/film	less 1 mr/film
Ritter		9–14 r/film		
Martin		1–15 r/film		
		av. 5 r/film		

dividual from such a dose of radiation to the chest. It could be predicted that for patients who have received many diagnostic examinations, both fluoroscopic and roentgenographic, there would be an appreciably increased incidence of leukemia and other neoplasms as well as a decrease in longevity.

A crude index of radiation dosage to the patient in common roentgenographic examinations is set forth in table 7. Great differences in dosage can result from small differences in filtration and voltage.

Physicians and radiologists should do everything possible to arrive at a diagnosis with a minimal amount of radiation. Here are some rules to follow:

Tend to use the more efficient higher kilovoltage and low milliamperes techniques.
Test your machine periodically for leakage and output.
Use at least 2 mm. of aluminum filtration.
Use field limiting cones and diaphragms as much as possible.
Choose the fastest practicable screen, film, and chemical combination.
Process films full time using active chemicals and proper temperature.

Fluoroscopy:

Adapt your eyes for twenty minutes before fluoroscoping.
Always wear protective gloves and aprons.
The fluoroscopic tube-table distance should be 18 inches or greater.
Keep the shutters down to the smallest practical field.
Use the fluoroscope quickly and efficiently to observe motion and function and not as a screening procedure. Do not day dream with foot on the switch.
Protect the gonads in patients, especially those less than forty.
Ask yourself, "Is this examination important to the health and well-being of the patient and what is the most efficient way to carry it out?"

BENEFITS FROM RADIATION

Thus far, we have been talking about the hazard of radiation. The picture, however, is incomplete without including some discussion of the other side of the coin, i.e., the benefits that can be derived from the proper use of radiation.

Throughout the history of mankind, it has been found that advances made through the use of energy and power have presented hazards. Then, when man could define, understand, and finally control this energy, he turned the hazards to his advantage.

The hazard of fire to the caveman was conflagration, wasting forests and animal life and endangering his own existence. When he could recognize and appreciate the potential of this energy, he harnessed the destroyer and made it his servant. It could then heat his cave, cook his food, and harden his clay into useful vessels.

Controlled explosions give us the combustion engine, which powers our motor cars and airplanes. These vehicles can be a menace or a blessing. They can and do cause death and destruction. Properly used, they broaden man's life and open up new horizons.

Lightning—electricity in the raw—is destructive. Electricity controlled gives us heat, light, power, radio, television, computers, and labor-saving devices of all sorts.

Nuclear energy, uncontrolled, produces a Nagasaki and Hiroshima and could conceivably destroy mankind. If man makes this source of energy his servant,

he can move a mountain, change the course of a river, and convert barren deserts to fertile gardens. He can probe Nature's subtlest secrets, such as the metabolic processes of life itself.

Harnessing of nuclear energy surpasses any of the challenges thus far presented to man. His experience of coping successfully with fire, electricity, and explosives should stand him in good stead in accepting this, his newest challenge.

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PROLONGED OBSERVATIONS OF PATIENTS WITH COR PUMONALE AND BULLOUS EMPHYSEMA AFTER SURGICAL RESECTION[1,2]

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Introduction

The surgical excision of pulmonary air cysts of various types has been an accepted procedure for many years (1–3). During this time several writers have stressed the variability of these lesions, both pathologically and physiologically, and the necessity for their precise definition in consideration of treatment (4, 5). Differing clinical and physiologic changes following surgical therapy of air cysts have been noted, although most reported studies have been concerned with alterations in pulmonary dynamics (4, 6). The present report is concerned with the description of some additional cardiovascular and hematologic changes in a small selected group of adults with long-standing diffuse obstructive emphysema, cor pulmonale, and large bullae, initially and after operative removal of the bullae.

An early and lucid pathologic description of the bulla and the bleb is contained in Thomas Watson's textbook on physic published in 1848 (7). In that voluminous storehouse of information, he describes air-containing pulmonary cysts "as large as hen's eggs," and others "equal to the size of the stomach of ordinary dimensions." Miller (8) later defined the differences between blebs and bullae, as seen in the cadaver (table 1). He stressed the importance of atrophy of the alveolar wall in the development of bullae. The tendency for local lung destruction and further expansion of bullae, once the basic lesion is established, has been noted (9, 10).

Materials and Methods

Six males and one female between fifty-one and sixty-nine years of age originally constituted the study group. The one postoperative death reduced this study group to 6 patients, all of whom have been followed for a minimum of one year after surgical ablation of large bullae. All give a history of chronic cough for many years with gradual increase in exertional dyspnea punctuated by frequent "chest colds." The males had been forced to stop work because of progressive pulmonary insufficiency. Three patients were in intractable cardiopulmonary failure at the time of operation.

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[2] Presented before the Medical Session, as part of *Section 3B*, at the annual meeting of the National Tuberculosis Association, Kansas City, Missouri, May 7, 1957.

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TABLE 1

BLEBS AND BULLAE OF ADULT EMPHYSEMA*

Bleb	Bulla
1. Less common in emphysema...responsible for mediastinal emphysema.	1. Common in emphysema....along lung margins.
2. Caused by rupture of alveolar wall → escape of air into alveolar layer of pleura → separation of pleura from alveoli.	2. Caused by gradual dilatation, thinning, and atrophy of alveoli → rupture with formation of bulla communicating with bronchial tree.
3. Outer wall formed by stretched pleura. Extends to boundary of septae.	3. Pleura remains in contact with greatly dilated alveoli.
4. Generally smaller...collapse with chest open...nonepithelized.	4. Project beyond lung surface...distended with chest open....nonepithelized....may become very large.

* William S. Miller—1926

The particular interest of the present writers was to study whether or not any operative procedure might benefit these "poor-risk" patients, all of whom had pulmonary hypertension and hypoxemia. The details of methods employed in studying this group of patients have been previously described and will only be summarized at this time (11–13). In addition to the customary clinical and roentgenographic studies, close attention was given to objective changes in the cardiac, pulmonary, and hematologic systems during the period of observation by repeating cardiac catheterization, pulmonary angiography, lung function studies, and determination of erythrocyte mass and blood volume. Residual lung volumes were not measured in these patients. All patients had an elevated mean pulmonary arterial pressure and hypoxemia at rest. Three showed a secondary erythrocytosis and hypervolemia at the time of operation.

During the period of observation these subjects were maintained on a regimen of oral and topical bronchodilators; antimicrobial drugs as needed for intercurrent respiratory infections; and digitalis, mercurial diuretics, and a low sodium diet when congestive heart failure was present. Cardiac catheterization was done when the subjects were free from respiratory infections and exhibited a steady clinical course.

The surgical procedure consisted of a partial excision of the bullae with suture of the adjacent wall and pleural surface to secure pneumostasis. No resection of adjacent lung tissue was done. Partial parietal pleurectomy was performed in 2 patients, but was later abandoned since excessive postoperative pleural space bleeding was encountered in these subjects. A tailoring thoracoplasty was required in one patient to obliterate a postoperative empyema.

RESULTS

Of these 7 "poor-risk" patients, one died of combined cardiopulmonary failure thirty-six hours following operation. He was the oldest (sixty-nine years). All of the 6 survivors experienced subjective improvement in the early months following operation. Three males were rehabilitated to the point of returning to gainful employment, and 2 are still working. One man developed a postoperative complication, an empyema which required a thoracoplasty. One patient died of cerebral hemorrhage sixteen months postoperatively.

Changes in ventilatory function, as measured by maximal breathing capacity and mean pulmonary arterial pressure of these patients, are listed in figure 1.

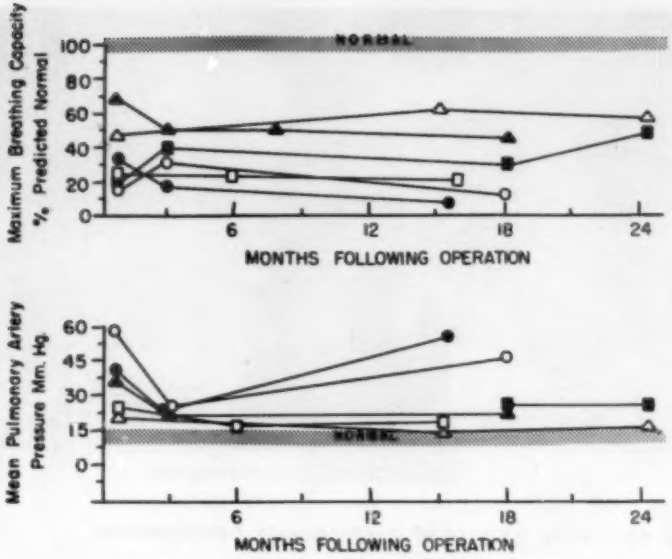


FIG. 1. Changes in cardiopulmonary function following operation.

In general, little significant change was noted in lung function either in the immediate or more remote postoperative periods. Pulmonary arterial pressure decreased immediately postoperatively in all patients, but later returned to preoperative values in some. The subjects with the most severe congestive heart failure and the highest pulmonary arterial pressure preoperatively exhibited the most striking reduction. As will be noted in figure 1, these were transient phenomena, for in later months the pulmonary arterial pressure returned to higher values.

Four patients will be discussed in greater detail to illustrate the variability presented by them.

Case 1. Progression of cardio-pulmonary disease: M. W., a 55-year-old Negro janitor, had a five-year history of progressive productive cough and dyspnea. Wheezing had been present for two years when he was first seen in 1955. In that year he had been forced to stop working because of dyspnea. Chest roentgenograms in figure 2 were obtained prior to operation, after the patient had been in congestive heart failure for two months. On admission to the hospital he was treated with digitalis, sodium restriction, and mercurial diuretics, with marked diuresis and a 10-pound weight reduction. Despite these measures, the patient later showed the severe cardiopulmonary abnormalities noted in figure 3. Thoracotomy was done in the hope of palliating his cardiopulmonary failure by surgical ablation of bullae in the right upper lung field. At operation the wall of a giant bulla approximately 12 cm. in diameter was partially excised, allowing its collapse. The adjacent lung was approximated and several smaller bullae were incised and sutured. The remaining portion of the right lung expanded to fill the right hemithorax.

The patient was less dyspneic following operation and returned to work after discharge from the hospital. His postoperative roentgenograms are shown in figure 4. He did well for several months but, as seen in figure 3, a relentless progression of pulmonary emphysema

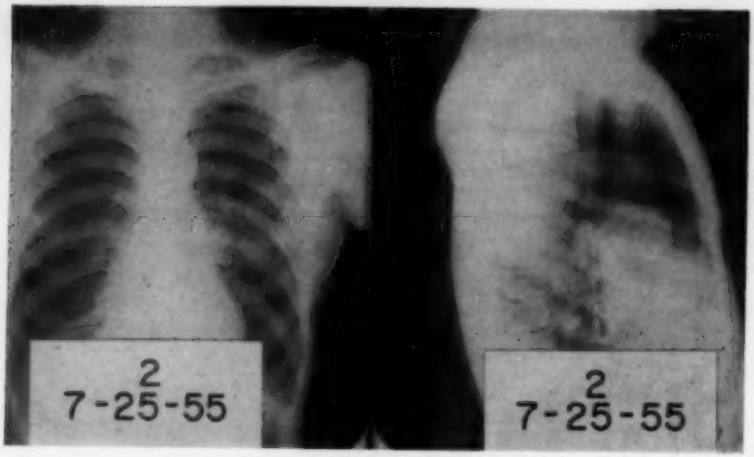


FIG. 2. *M. W.*, preoperative roentgenograms.

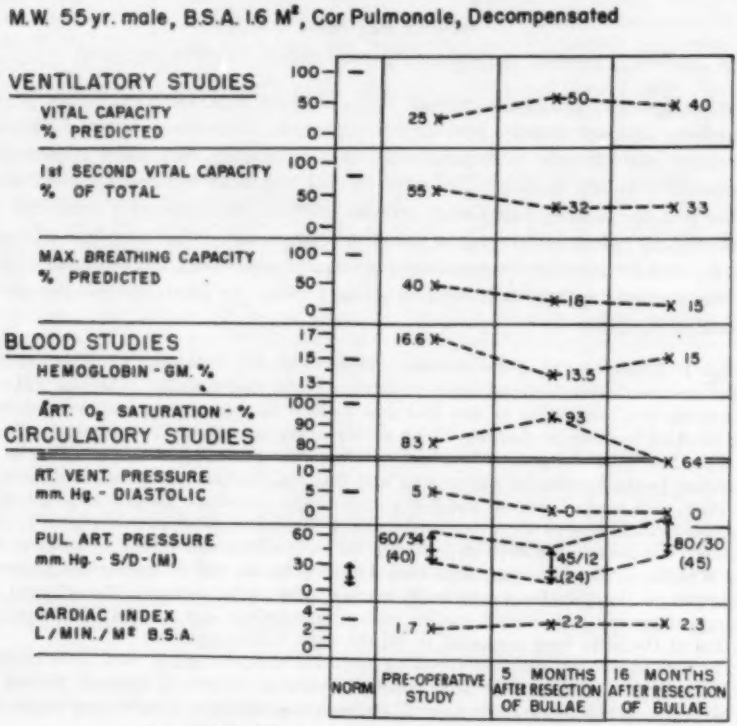


FIG. 3. *M. W.*, summary of physiologic data.

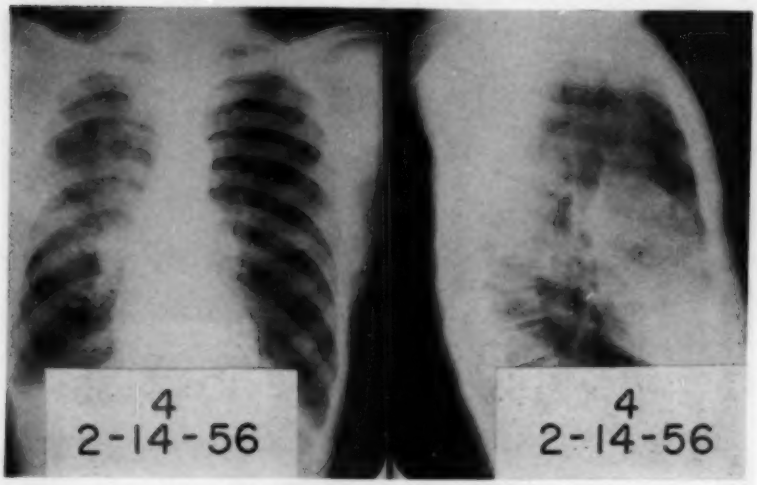


FIG. 4. *M. W.*, postoperative roentgenograms.

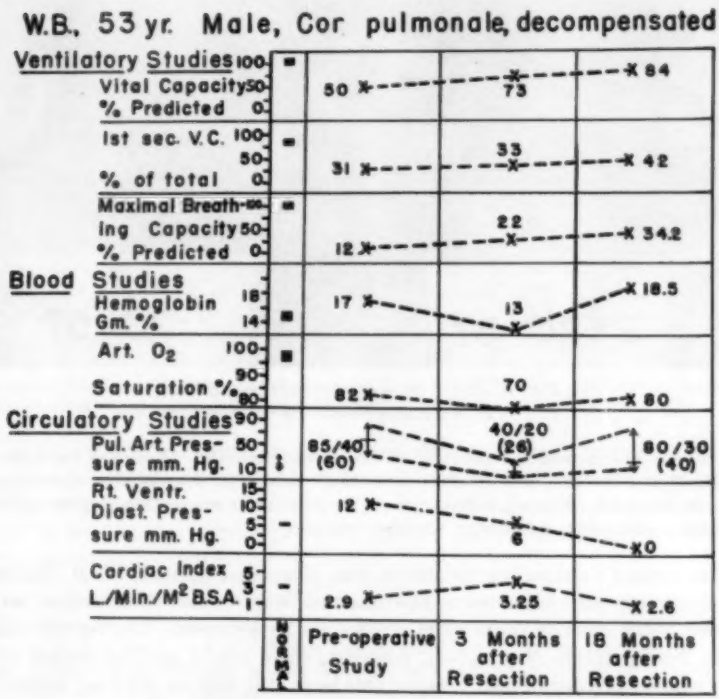


FIG. 5. *W. B.*, summary of physiologic data.

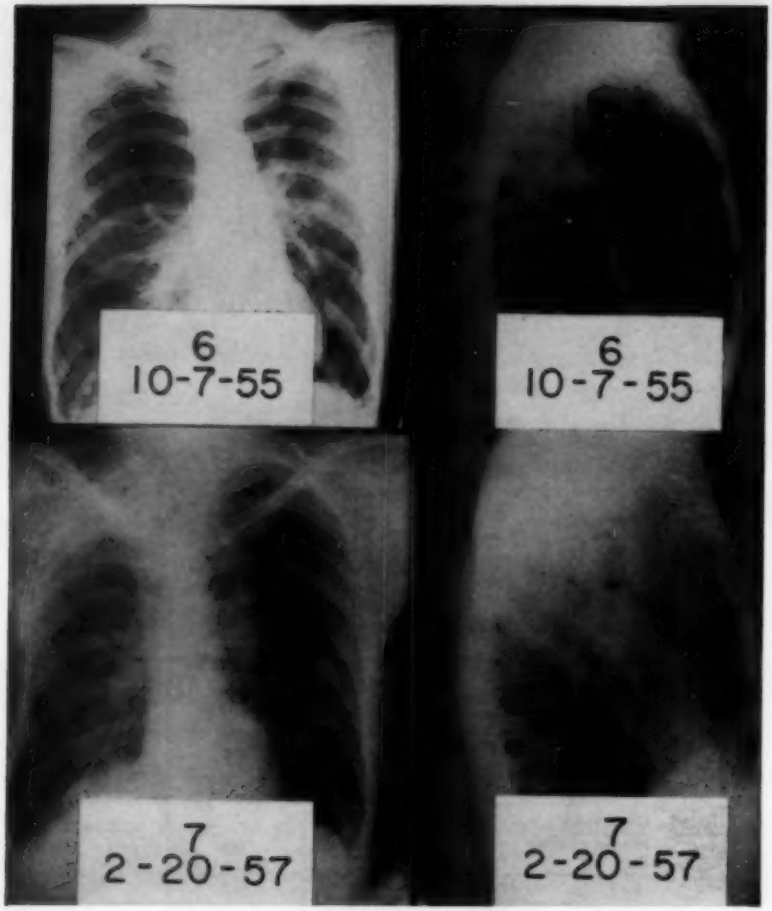


FIG. 6. *A. C.*, preoperative roentgenograms.
FIG. 7. *A. C.*, postoperative roentgenograms.

and cardiac decompensation occurred in the ensuing months. Increasing cardiomegaly developed and he died of a cerebral hemorrhage seventeen months after thoracotomy. Autopsy disclosed advanced pulmonary emphysema, right ventricular hypertrophy and dilatation, and severe pulmonary vascular sclerosis.

This patient's course was similar to that of another patient, *W. B.* (figure 5). The latter was also subjected to operation while in cardiac decompensation and showed a similar early lowering of pulmonary hypertension. During the months of his postoperative observation, however, there was a gradual return to the values noted preoperatively. Despite this he is still able to work on limited activity.

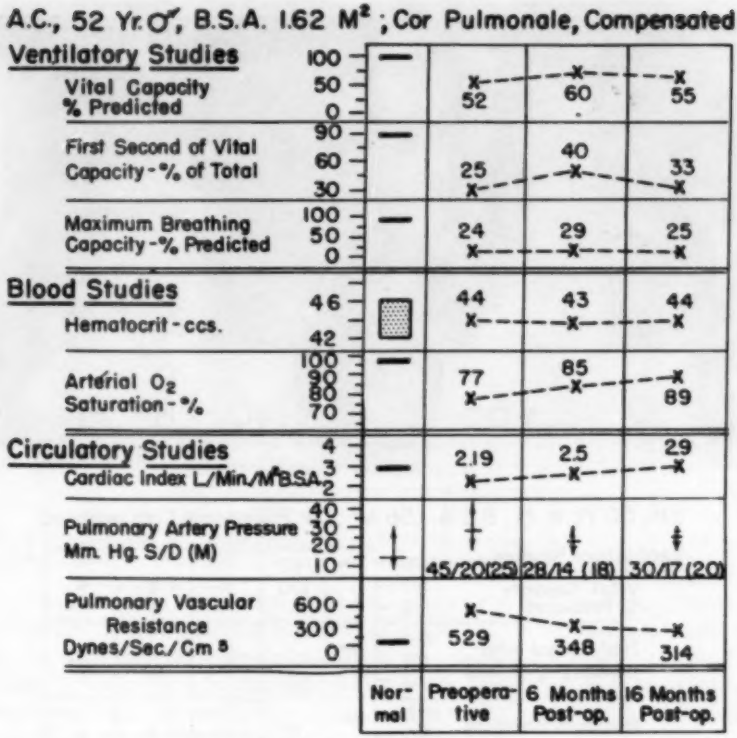


FIG. 8. *A.C.*, summary of physiologic data.

Case 2. Stabilization of cardiopulmonary disease: A. C., a 52-year-old white farmer, was first studied for chronic pulmonary disease in 1955. He had noted a chronic cough with increasing exertional dyspnea of four years' duration and had stopped working in 1953 because of dyspnea. Roentgenograms (figure 6) showed advanced emphysema with bullae in the right upper lung field. Thoracotomy was done, disclosing several bullae up to 10 cm. in diameter. These were incised with partial excision and suture of their walls. The remaining lung expanded to fill the right hemithorax. He was subjectively improved postoperatively and has remained so (figure 7).

The data in figure 8 reveal no appreciable change in this patient's pulmonary dynamics or erythrocyte mass. A sustained decrease in hypoxemia and resting pulmonary arterial pressure along with an increase in cardiac output should be noted. At present this man has a better exercise tolerance, despite no detectable improvement in his ventilation.

Case 3. Bullectomy and thoracoplasty: G. H., a 56-year-old farmer, dated his chronic cough and dyspnea to 1952. Chest roentgenograms and angiogram (figure 9) showed large bullae in the left upper lung field with a marked reduction in the vascular pattern in that region. Thoracotomy disclosed several large bullae originating from the left upper lobe. The bullae were incised, their walls partially excised, and the lung was sutured. Parietal pleurectomy was done. The patient's postoperative course was complicated by severe

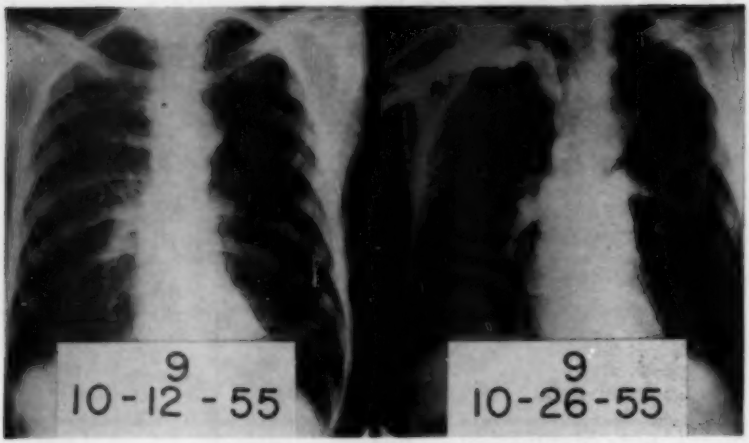


FIG. 9. *G. H.*, preoperative roentgenograms.

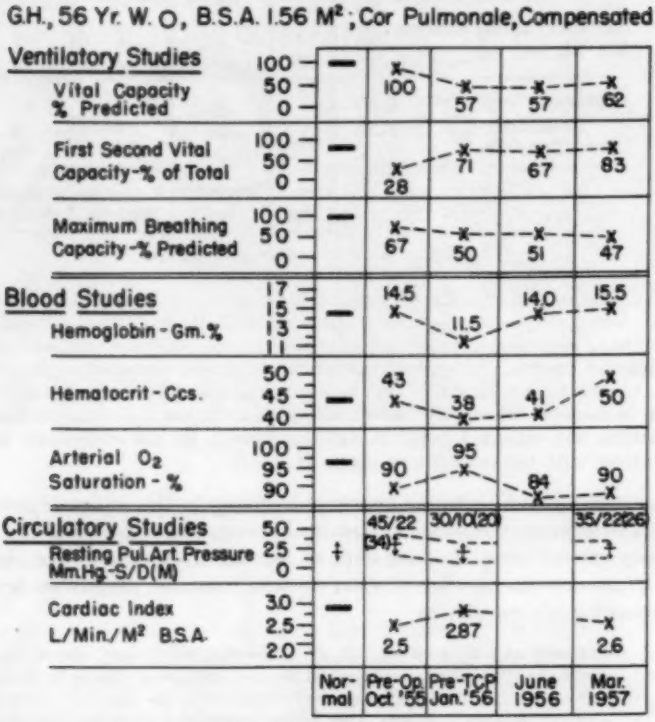


FIG. 10. *G. H.*, summary of physiologic data.

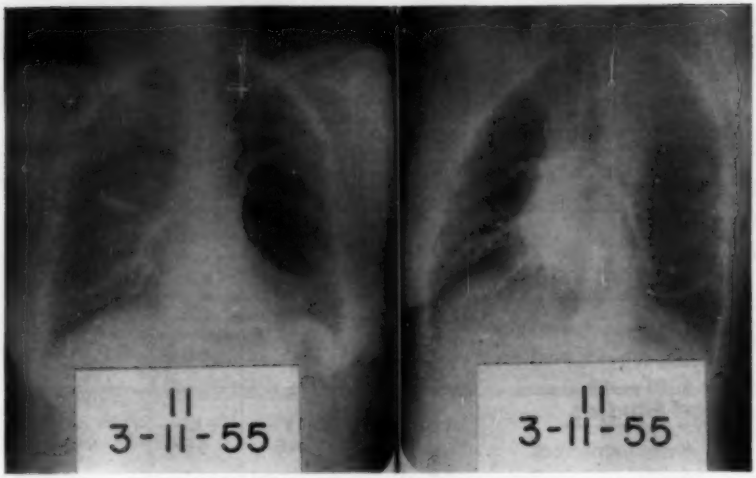


FIG. 11. *L. Z.*, preoperative roentgenograms.

pleural bleeding and the subsequent development of an empyema. A tailoring thoracoplasty was done three months following his first thoracotomy.

The data listed in figure 10 show an improvement in this man's ventilation, as evidenced by a rise in the first-second vital capacity despite a decrease in thoracic size. An early reduction in pulmonary hypertension with a later rise and an increase in the erythrocyte mass should be noted. At present the patient is much less dyspneic than preoperatively and has exhibited little progression of emphysema during the period of observation.

Case 4. Excision of an epithelized cyst: L. Z., a 66-year-old housewife, was first seen in 1955, complaining of exertional dyspnea of two years' duration. She had noted an increasing chronic cough and was producing a maximum of one cup daily of mucoid nonpurulent sputum. Intermittent bilateral wheezing had also been noted in the past year. Chest roentgenograms and bronchograms disclosed a large centrally located air-containing cyst without bronchial communication (figure 11). Bronchoscopy disclosed narrowing of the left lower bronchus and diffuse hyperemia of the mucosal surface.

Left thoracotomy was done, and a large thick-walled cyst in the area of the major fissure was found and excised. No bronchial communication was demonstrated. The cyst subsequently proved to be ciliated and bronchogenic with a well-differentiated epithelial lining. Postoperatively the chest roentgenogram showed complete re-expansion of the left lung to fill the hemithorax.

The patient was subjectively improved by operation and this improvement has continued to the present time. She still has a slight cough with intermittent periods of wheezing, but these are readily controlled by bronchodilators. As is noted in figure 12, no significant change in her pulmonary circulation during this period of observation was noted, although there has been a mechanical improvement in her lung function and in arterial oxygen saturation.

This patient demonstrates a different problem from those previously described. She had an epithelized developmental lung cyst rather than atrophic

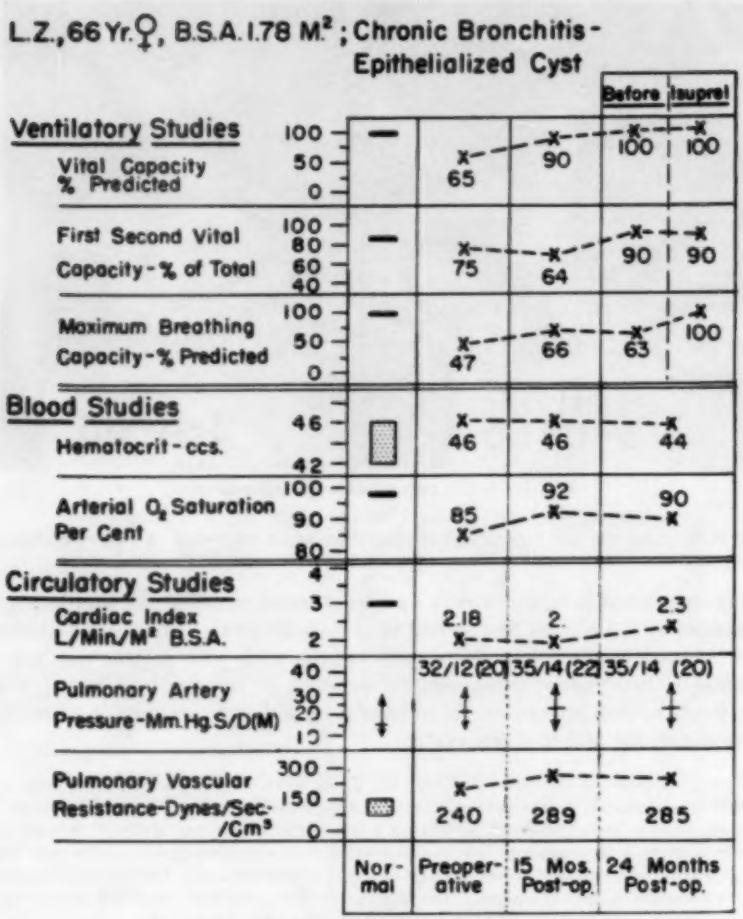


FIG. 12. *L. Z.*, summary of physiologic data.

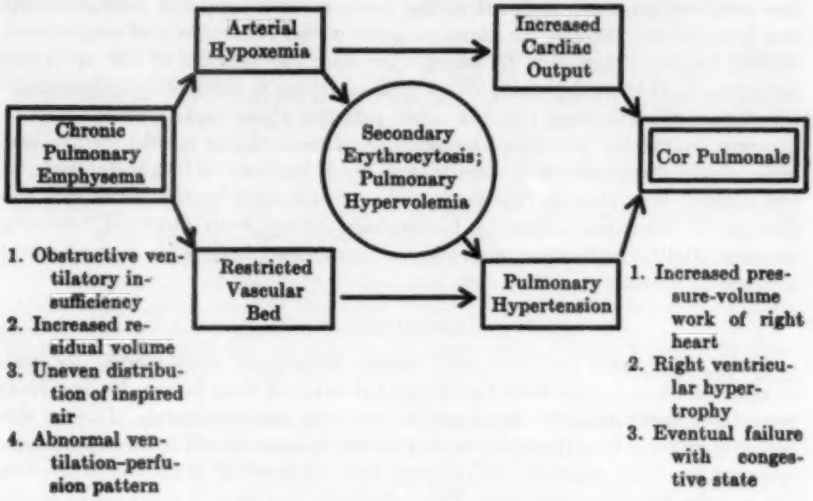
bullae of emphysematous origin. Her dyspnea was associated with intermittent bronchial narrowing and was amenable to medical therapy. Her ventilation has improved postoperatively due to expansion of a previously collapsed lung without change in the pulmonary arterial pressure.

DISCUSSION

In considering possible benefits from a surgical attack in patients with diffuse obstructive emphysema, large bullae, and cor pulmonale, the natural evolution of this disease picture must be carefully considered. In recent years knowledge of the fundamental physiologic and pathologic processes involved has been

TABLE 2

FACTORS CONTRIBUTING TO DEVELOPMENT OF COR PULMONALE



enriched by the studies of Baldwin, Cournand, Richards, and their co-workers (4, 14–16).

In simple form it may be said that pulmonary emphysema, a predominantly degenerative pulmonary disease of aging males, is most frequently associated with chronic bronchitis. Its course is usually spread over many years with a gradual and progressive reduction of pulmonary reserve. As shown in table 2, the resultant abnormal lung volumes, the obstructive pattern of air flow, the increased work of breathing, and the abnormal ventilation–perfusion relationships are frequently associated with a gradual anatomic reduction in capacity of the normal voluminous capillary bed and, in some patients, with the development of fixed pulmonary hypertension with secondary cardiovascular and hematologic changes. These lead to a readjustment of the right heart to an increased work load to maintain normal flow through this restricted vascular bed. Intercurrent bronchial infections with associated augmentation of hypoxemia further accentuate these abnormalities and contribute ultimately to cardiac hypertrophy with dilatation and failure of the right heart.

The results of therapy at any phase of this protracted course might be detectable by objective changes in the pulmonary, cardiovascular, or hematologic findings of the patient. However, the prolonged time required for development of this end-stage of cardiopulmonary failure, the irreversible nature of many of the anatomic changes, and the frequent overlapping of these entities often make it difficult or impossible to detect or measure therapeutic effect.

In the majority of the patients reported here, no significant improvement was

noted in ventilation following operation. This is compatible with the protracted course and the diffuse nature of emphysema. Some objective benefits from operative intervention were observed in the more recently acquired cardiovascular and hematologic changes. In those patients whose bronchitis and emphysema showed further progression following operation, the benefits of the operation proved to be of short duration, with a gradual return to preoperative abnormalities during the following year. In other patients whose basic disease failed to progress as rapidly following operation, reversion of the cardiovascular and hematologic abnormalities to more normal levels has been of longer duration. In one patient with chronic bronchitis and an epithelized bronchogenic cyst, no changes in cardiopulmonary or hematologic values were observed following excision. Her symptomatic improvement correlated best with a mechanical increase in ventilation.

Summary

Seven "poor-risk" patients with chronic pulmonary emphysema, cor pulmonale, and large bullae have had surgical ablation of their bullae. Six survivors have been followed for a minimum of one year postoperatively. During this period changes in lung function, cardiovascular dynamics, and in the hematologic system have been observed. All patients were subjectively improved in the first few months following operation. This correlated best with changes noted in the circulatory system. Progression of emphysema was observed later in some patients following transient improvement associated with surgical therapy.

Sumario

Observaciones Prolongadas de Enfermos de Corazón Pulmonar y Enfisema Vesicular Después de la Resección Quirúrgica

En 7 enfermos de enfisema pulmonar crónico, corazón pulmonar y vesículas grandes, se ejecutó la ablación quirúrgica de las vesículas. A 6 sobrevivientes se les ha mantenido postoperatoriamente en observación a lo menos por un año. Durante este período se han observado alteraciones de la función pulmonar, de la dinámica cardiovascular y del aparato hematológico. Todos los enfermos mejararon subjetivamente en los primeros meses consecutivos a la operación. Esto correlacionó mejor con las alteraciones notadas en el aparato circulatorio. En algunos enfermos se observó más tarde agravación del enfisema después de la mejoría pasajera vinculada con la intervención cruenta.

Resume

Observations prolongées de malades présentant un coeur pulmonaire et un emphysème bulleux. Après résection chirurgicale

Sept malades, considérés comme de "mauvais cas", atteints d'emphysème pulmonaire chronique à large bulle avec coeur pulmonaire, ont subi l'exérèse de ces bulles. Six survivants ont été suivis pendant un minimum d'une année après l'intervention. Au cours de cette période des changements ont été observés dans la fonction pulmonaire, la dynamique cardio-vasculaire et le système hématologique. Du point de vue subjectif, tous les malades ont été améliorés dans les premiers mois qui suivirent l'intervention. Ceci coïncidait particulièrement avec les modifications observées dans le système circulatoire. La progression de l'emphysème a été observée ultérieurement chez certains malades, après une amélioration passagère en rapport avec le traitement chirurgical.

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LUNG FUNCTION IN SILICOSIS OF THE WITWATERSRAND GOLD MINER[1, 2]

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INTRODUCTION

One of the first studies of respiratory function in classical silicosis was that of Dowds, who investigated 500 Rand gold miners with varying roentgenographic grades of the disease (1). He showed that with roentgenographic progression of the disease there was a progressive reduction of vital capacity. The volume of resting ventilation, however, did not change significantly although the frequency increased and the tidal volume decreased. Subsequently studies from many centers have established the other features of lung function in this condition.

In the stage of diffuse nodulation it is usual to find a reduction of the total lung volume (2–7) due to the reduction in vital capacity (1–8). Residual volume is usually reported as normal or slightly decreased (3–6), although in two studies it was found to be increased (2, 3). Although disturbances of intrapulmonary mixing are considered to be unusual in diffuse pulmonary fibrosis by some workers (4), uneven intrapulmonary mixing has been reported in nodular silicosis (5, 7). Maximal breathing capacity is generally thought to be reduced (4, 6, 7), as are the so-called "indirect" tests of maximal breathing capacity (10). Most workers report an increase in exercise ventilation (3, 4, 11). Bruce (7), however, finds no significant change until the disease becomes conglomerate. A fall in the arterial oxygen saturation on effort is commonly reported (2, 8, 12).

The first object of this study was to determine the pattern of disturbance of respiratory function in the silicosis of the Witwatersrand gold mines in greater detail than had been done by Dowds (1). A second aim was to correlate physiologic disturbances with the roentgenographic pattern. Lack of correlation between clinical and physiologic status, on the one hand, and roentgenographic status, on the other hand, has been frequently reported in classical silicosis (2, 5, 8, 13). Most studies, however, have been carried out on subjects under consideration for compensation, and do not constitute a cross-section of all men employed in the particular industry concerned. By contrast the unique study reported by Gilson and Hugh-Jones (14) was based on random selection from all subjects in any one roentgenographic category in the Welsh coal mining industry, irrespective of the presence of symptoms. It thus defined the incidence and degree of disturbance of function in a particular roentgenographic category of coal

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miners' pneumoconiosis. It was hoped that the present study would supply the same information for classical silicosis as is seen in the Witwatersrand gold mines.

Plan of the Study

The basic plan of the experiment was similar to the study of the Cardiff workers (14) with modifications. Only two roentgenographic groups were studied: miners whose chest roentgenograms were normal and miners whose chest roentgenograms showed marked discrete nodular silicosis widely distributed throughout both lungs. Intermediate groups were not studied.

In order to define the population to be studied, certain criteria for selection of cases were applied beforehand. Thus subjects were excluded who had had less than seven years' service underground or who had had more than six months in any other type of mine than those of the Witwatersrand geologic complex.

The chest roentgenograms of all men examined at the Pneumoconiosis Bureau during the period December, 1955, to May, 1956, were classified according to the radiologists' interpretation (15). This annual physical and roentgenographic examination is compulsory for all working miners in South Africa, and it was therefore thought that the groups so defined roentgenographically would be as nearly random samples of all men in the industry as possible.

The plan of the study is illustrated below:

Age	Roentgenographic Category, Normal Chest Pattern	Roentgenographic Category, Generalised Small or Medium Nodulation
years		
41–50	10 cases (*315*)	10 cases (*34*)
51–60	10 cases (*161*)	10 cases (*51*)

The number in brackets is the total number of cases in the particular population from which the sample was drawn at random. Certain cases had to be replaced after sampling for the following reasons:

a. Inadequate service or mixed mining service (*see* above) not recorded on the roentgenographic check-card, and thus the case had not been excluded before sampling.
b. Severe complicating injury, hemiplegia following a rock-fall injury to skull.
c. Complicating features on the chest roentgenogram e.g., egg-shell calcification of hilar lymph nodes, large dense calcified foci.
d. Residence more than 300 miles from Johannesburg.

Techniques Used

Vital capacity, maximal midexpiratory flow (16), and maximal breathing capacity of more than 15 seconds were measured on a Lode spirometer checked to record volume accurately without overshoot at the frequencies used by the subjects (up to 120 per minute). Functional residual volume was measured by the closed-circuit constant-volume method of McMichael (17) with certain adaptations, and a mixing index was calculated from the time taken for equilibrium to be reached between the lungs and the spirometer (18). Values for lung volume and its subdivisions were compared with values predicted from various body characteristics (19).

The work of breathing was studied by the method of Mead and Whittenberger (20), pressure changes being recorded on a straingauge manometer; airflow, on a Lilly pneumotachygraph screen with Lilly manometer; and tidal volumes, again on a Lode spirometer. Compliance (liter per cm. water pressure) and nonelastic breathing resistance (cm. water

pressure per liters per second) were calculated at rest, at high breathing frequencies, and after exertion; the mean resting end expiratory pressure was noted.

Minute ventilation and oxygen uptake were studied during an exercise step test at 3 to 4 different speeds. Minute ventilation was recorded on a Lode spirometer filled with air, while oxygen uptake was recorded by the movement of a second spirometer bell acting as a compensating circuit (21). By plotting minute ventilation against oxygen uptake at the different work loads, a graph was drawn from which the ventilation at 1 liter of oxygen uptake could be derived. This could be compared with the ventilation equivalent for oxygen at rest. Some subjects carried out the Hugh-Jones step test as well (22).

During the exercise studies, arterial oxygen saturation was measured by a Waters-Conley absolute reading ear oximeter, and after exercise the rise of saturation on breathing 100 per cent oxygen was noted.

Thus in all, twenty results were calculated on each patient:

1. Vital capacity in liters (VC)
2. Vital capacity as per cent of predicted value
3. Residual volume in liters (RV)
4. Residual volume as per cent of predicted value
5. Total lung capacity in liters (TLC)
6. Total lung capacity as per cent of predicted value
7. Functional residual capacity in liters (FRC)
8. Functional residual capacity as per cent of predicted value
9. $\frac{\text{FRC}}{\text{TLC}} \times 100\%$
10. $\frac{\text{RV}}{\text{TLC}} \times 100\%$
11. Maximal midexpiratory flow (liters per second)
12. Mixing index (liters per liter FRC)
13. Ventilation equivalent at rest (liters per 100 cc. oxygen uptake)
14. Ventilation equivalent at an exercise requiring one liter of oxygen uptake per minute
15. Fall of ventilation equivalent on effort
16. Compliance at rest (liters per cm. water pressure)
17. Compliance at high breathing frequency
18. Expiratory nonelastic resistance (cm. water per liter per second)
19. Fall in arterial oxygen saturation on effort
20. Rise in arterial oxygen saturation on pure oxygen breathing
21. The sum of the maximal fall in arterial saturation on effort, i.e., *Test 19*, plus the maximal rise in arterial saturation on pure breathing oxygen, *Test 20*.

Results

The individual test results are reflected in tables 1 and 2.

Statistical analysis confirmed the impression gained by scrutiny of the results that no single test discriminated satisfactorily between miners with normal chest roentgenograms and those with advanced silicosis.

Accordingly, a discriminant analysis was carried out to see if any group of tests appropriately combined might not effect separation between these groups. The twenty test results were examined for intercorrelation. Eight were then selected on two criteria: first, that their results did not interrelate with one another to a statistically significant degree; second, that they were not measuring similar aspects of function from the physiologic point of view. The tests thus chosen were numbers *2*, *8*, *11*, *12*, *14*, *16*, *18*, and *21* from the above list.

TABLE 1

RESULTS OF LUNG VOLUME STUDIES, MIXING, AND EXERCISE VENTILATION

	Vital Capacity	Per Cent Predicted	Residual Volume	Per Cent Predicted	Functional Residual Capacity	Per Cent Predicted	$\frac{RV}{TLC} \times 100$	$\frac{FRC}{TLC} \times 100$	Mixing Index	Oximetry		Ventilation Equivalent for Oxygen		Hugh Jones Effort Test		
										Maximal Fall on Effort	Maximal Rise on Oxygen	At Rest	On Effort	Exercise Ventilation	Standard Ventilation	Dyspneic Index
Miners with no silicosis shown roentgenographically																
Aged 41–50	4.831	106.1	1.928	94.2	3.513	101.0	28.20	51.60	3.330	−1.34	6.30	4.35	2.99			
Standard deviation	0.753	15.0	0.507	25.4	0.737	20.4	18.19	8.97	0.558	1.77	1.38	1.42	0.56			
Aged 51–60	4.302	113.5	2.046	99.4	3.242	103.8	32.10	49.70	3.848	+0.02	5.56	4.57	3.16	33.90	35.30	30.90
Standard deviation	0.633	14.0	0.368	14.4	0.709	17.8	13.34	7.32	0.548	2.89	1.77	1.74	0.58	3.32	9.93	20.40
Miners with marked silicosis shown roentgenographically																
Aged 41–50	4.388	97.2	2.087	100.3	3.280	95.7	31.80	50.90	3.710	−2.07	6.13	5.77	3.51			
Standard deviation	0.850	15.7	0.572	23.6	0.779	15.0	24.80	8.09	0.645	2.08	2.18	1.94	0.72			
Aged 51–60	3.620	89.4	2.290	104.6	3.360	98.9	37.80	56.10	4.584	−1.04	4.56	5.24	3.39	35.95	37.55	54.00
Standard deviation	0.550	13.9	0.794	31.3	0.878	21.1	27.68	8.32	0.852	1.55	1.30	1.31	0.33	5.89	5.14	7.30

TABLE 2

RESULTS OF WORK OF BREATHING STUDIES

	Maximal Breathing Capacity	Per Cent Predicted	Maximal Midexpiratory Flow	At Rest				Fall in Compliance on Hyperventilation f* ± 50 per min.	After Exertion				Resting End Expiratory Pressure
				Compliance	Resistance Inspiration	Resistance Expiration	Breathing Frequency per Minute		Compliance	Resistance Inspiration	Resistance Expiration	Breathing Frequency per Minute	
Miners with no silicosis shown roentgenographically													
Aged 41–50	147.4	106.7	3.12	0.234	2.67	3.02	19.9	−0.015	0.221	2.005	2.879	24.4	−3.550
Standard deviation	33.5	28.9	1.06	0.052	0.77	0.89	4.7	0.044	0.049	0.864	0.746	4.5	0.989
Aged 51–60	119.9	104.9	2.38	0.199	3.22	4.39	16.9	−0.042	0.179	2.900	2.753	22.0	−4.580
Standard deviation	29.8	25.3	0.91	0.044	0.84	1.06	23.3	0.057	0.050	1.131	1.180	6.0	1.696
Miners with marked silicosis shown roentgenographically													
Aged 41–50	128.7	96.9	2.12	0.194	2.74	4.06	20.6	−0.045	0.174	2.020	3.075	26.2	−3.780
Standard deviation	39.3	31.1	0.91	0.063	0.89	1.55	3.1	0.043	0.044	0.572	1.164	7.7	1.904
Aged 51–60	80.4	68.5	1.47	0.172	3.21	4.03	20.0	−0.029	0.164	2.520	3.332	29.3	−3.690
Standard deviation	37.3	34.0	0.77	0.066	1.96	1.44	5.2	0.063	0.061	1.164	1.283	6.1	1.578

*f = breathing frequency per minute.

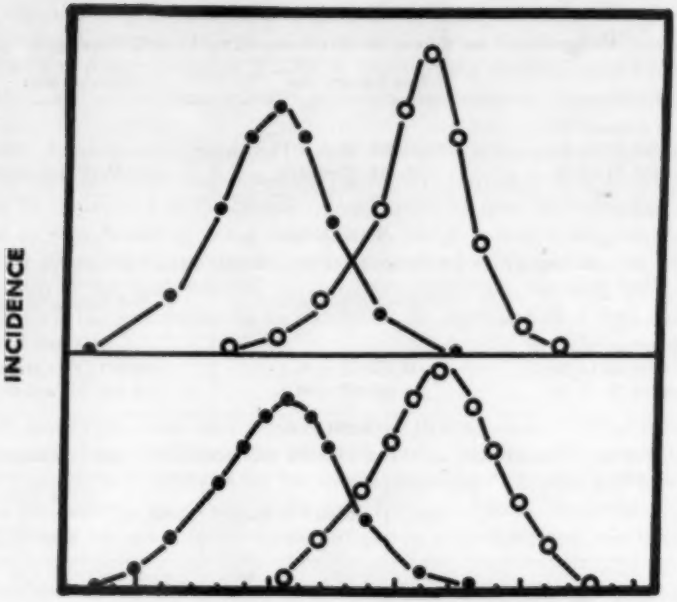

FIG. 1. Efficiency of lung function score in separating miners with normal chest roentgenograms from miners whose chest roentgenograms show diffuse nodular silicosis.
Top graph: men between the ages of 41 and 50 years
Lower graph: men between the ages of 51 and 60 years
Closed circles: men with normal chest roentgenograms
Open circles: men with diffuse nodular silicosis on chest roentgenograms

By means of the technique of discriminant analysis, weights were calculated for each of the eight selected tests to maximize differences between the two defined groups of cases. The test results each multiplied by its appropriate weight could then be added together to give a total physiologic test score. The degree of separation between the two roentgenographic groups achieved by this score is indicated in figure 1, and it may be seen that in either direction an overlap of about 15 per cent occurs. It should be noted that the form of the graph is derived from the means and variances of the total function scores, assuming a normal distribution (23), the cases in the present study being too few to represent by histogram.

The results were next studied to see if fair discrimination between the two roentgenographic groups of cases could be achieved with fewer tests; in other words, if any two or three of the tests used above could be employed as a screening procedure for silicosis. As has been mentioned above, no one of the twenty tests applied was shown to discriminate satisfactorily on its own. By the addition

TABLE 3

CORRELATION OF GRADE OF DYSPNEA WITH LUNG FUNCTION

	Lung Function Score	Dyspneic Index
Grade of dyspnea in		
men aged 41 to 50	−0.269 ($P < 0.1$)	—
men aged 51 to 60	−0.344 ($P < 0.1$)	+0.636 (P 0.01–0.001)

TABLE 3A

CORRELATION OF LUNG FUNCTION SCORE WITH SERVICE

	Total Service Underground	Rock-breaking Service
Lung function score in		
men aged 41 to 50	−0.151 ($P < 0.1$)	−0.681 ($P > 0.001$)
men aged 51 to 60	−0.056 ($P < 0.1$)	−0.556 ($P > 0.01$)

TABLE 4

PHYSICAL CHARACTERISTICS AND MINING SERVICE OF MINERS STUDIED

	Age	Height	Weight	Surface Area	Mining Service (years)	
					Total	Rock-breaking
	years	*cm.*	*kg.*	*sq. ml.*		
Miners with no silicosis shown roentgenographically						
Aged 41–50	45.00	175.60	78.50	1.9360	17.00	4.60
Standard deviation	10.75	5.80	11.40	0.1637	3.07	5.77
Aged 51–60	54.80	169.00	74.40	1.8480	21.50	3.30
Standard deviation	9.98	4.59	7.62	0.1056	6.08	4.29
Miners with marked silicosis shown roentgenographically						
Aged 41–50	46.70	174.70	78.90	1.9380	17.90	9.50
Standard deviation	6.99	5.20	13.13	0.1454	4.72	4.19
Aged 51–60	54.70	173.60	76.90	1.9050	21.60	11.60
Standard deviation	9.30	4.58	8.11	0.1080	5.89	6.09

of one further test at a time, the discriminating "distance" between the two groups was calculated, using Rao's method of discriminating analysis (24). Results showed that in men between forty-one and fifty years of age, all of the eight tests must be included before the "distance" is of any discriminatory value; for the age group fifty-one to sixty years, six tests must be used to get comparable separation. In other words, no good evidence could be found in the present studies for the use of less than the eight tests mentioned as a screen procedure for silicosis.

The relationship between symptoms and the lung function score was next examined. Dyspnea, assessed by careful questioning of the patient and graded

0 to 4 (22), did not correlate significantly with the lung function score ($P < 0.1$) although it did correlate with the index calculated from the Hugh-Jones exercise test (P 0.01 to 0.001) as shown in table 3. Neither lung function score nor Hugh-Jones dyspneic index correlated with a clinical assessment of the man's working capacity.

Finally, it was noted that of the 20 men with silicosis shown roentgenographically, 19 had worked on rock-breaking for various periods of their underground service. In contrast, 7 of the 20 roentgenographically normal miners had never worked on rock-breaking, being employed on assay, survey, fitting, timbering, and other jobs of that variety, which are probably less dusty. Accordingly the relationship of the lung function score to service on pneumatic drills was examined, and this was shown to be significant (> 0.01 and > 0.001) (*see also* tables 3.4, and 4).

Discussion

Dowd's study on Rand mine workers made in 1930 indicated a fall in the mean vital capacity from 4.09 liters for 100 long-service miners with no roentgenographic changes to 3.610 liters for 100 men with nodular silicosis. The mean age for the two groups was thirty-eight and forty-two years, respectively. The present figures are not directly comparable since techniques and selection were different.

It was interesting, however, to note in the forty-one to fifty-year age group a mean value of 4.83 liters for men with no roentgenographically demonstrable disease and 4.39 liters for men with nodular silicosis. This observation is in keeping with the clinical impression that the Rand disease is more benign now than it was in the days of Dowd's work.

The data were next examined to see if the pattern of functional disturbance shown in Rand miners with silicosis was similar to the pattern described in cases of classical silicosis from elsewhere. Various lung function tests were considered individually. Vital capacity was reduced in both age groups studied, and residual volume was increased. These changes, admittedly not significant in the statistical sense (table 1), are nevertheless directionally similar to those reported by other workers (2, 5–7). Nevertheless, the degree of change shown here was less marked than in other reports (2, 5). It seems possible that this difference is due to the fact that the patients in the other series were selected because of disability or suspected disability, whereas the patients of the present study were selected at random from workers in the industry. It should also be borne in mind that, despite the efforts made by the present workers to study cases selected at random from the industry, the group of men with nodular silicosis may be biased in favor of the more healthy subject. This is because of the tendency in recent years for men with nodular silicosis to be advanced to full compensation if they are disabled, although their roentgenographic findings have not advanced to conglomeration. Once fully compensated, such persons do not attend the Bureau for regular examination. It is the impression of the present workers that this bias is not considerable, although of course it is impossible to be certain of its extent.

Uneven intrapulmonary distribution of inspired air appears to be a feature of the advanced nodular silicotic in the present series, although again the changes are not of a degree which is significant statistically. This finding is in keeping with the reports of Roelsen and Bay (5), who used a different technique.

The mean ventilation equivalent for oxygen at rest and on effort was raised in the men with nodular silicosis in the present study. However, the changes were not of an order suggesting a statistically significant difference from the men with no roentgenographic change. Kaltreider and his co-workers (3) also found a high ventilation equivalent for oxygen, both at rest and on effort, in eleven cases of industrial silicosis, but the changes were more marked than in the present study. The present findings accord more closely with those of Roelsen and Eskildsen (11), who showed a slight increase in ventilation of silicotic subjects for a standard exercise (400 kg. per min. for 10 minutes). It should be noted that the absolute values for ventilation equivalent obtained in this laboratory (5,670 feet above sea level) are not comparable with those quoted by Roelsen and Eskildsen (working at sea level) or by Kaltreider and his colleagues in Rochester, New York (about 500 feet above sea level).

Thus it appears that lung function disturbances in Rand gold miners (as appraised by measurements of lung volume, intrapulmonary mixing, and exercise ventilation) are similar to those reported in subjects with classical silicosis from Sweden (5, 7) and the United States (2, 4).

Comment should be made on the manner in which the results in the present study compare with those of Rossier and his colleagues on 400 cases of silicosis from different industries in Switzerland (at an altitude of 3,000 feet). By contrast with the present study, their investigation was orientated to attempt exact assessment of the disturbance in the subject's capacity for exertion.

Using Rossier's criteria of disability based on the level of arterial oxygen at rest and after effort, all but 2 of the 40 cases studied in the present series would fall into the group of "latent insufficiency." Furthermore, since no significant fall in arterial saturation was observed on a Hugh-Jones effort test (load 350 kg. per min., equivalent to 58.4 watts), all patients in the present series would be considered to have retained at least one-third of their working capacity. Unfortunately, subjects in the present series were not studied at higher work loads than 58.7 watts; hence there is no means of estimating more closely how they fit into Rossier's classification.

The overlap of lung function scores between the two roentgenographic groups in our series was approximately 15 per cent. This figure is remarkably close to that of Rossier (8) who reported that 13 per cent of patients with no roentgenographic change had significant functional disturbance, and 18 per cent of men with marked nodular silicosis had normal function. In Roelsen and Bay's series (5) of 13 *stage 2* silicosis patients, the function test of one only was judged to be essentially better than the roentgenographic findings. The findings in the present study are thus in agreement with those of both the Swiss (8) and Scandinavian workers (5).

Since the study on Welsh coal miners (14) and the present study on Rand gold miners were both based on random selection of miners throughout the

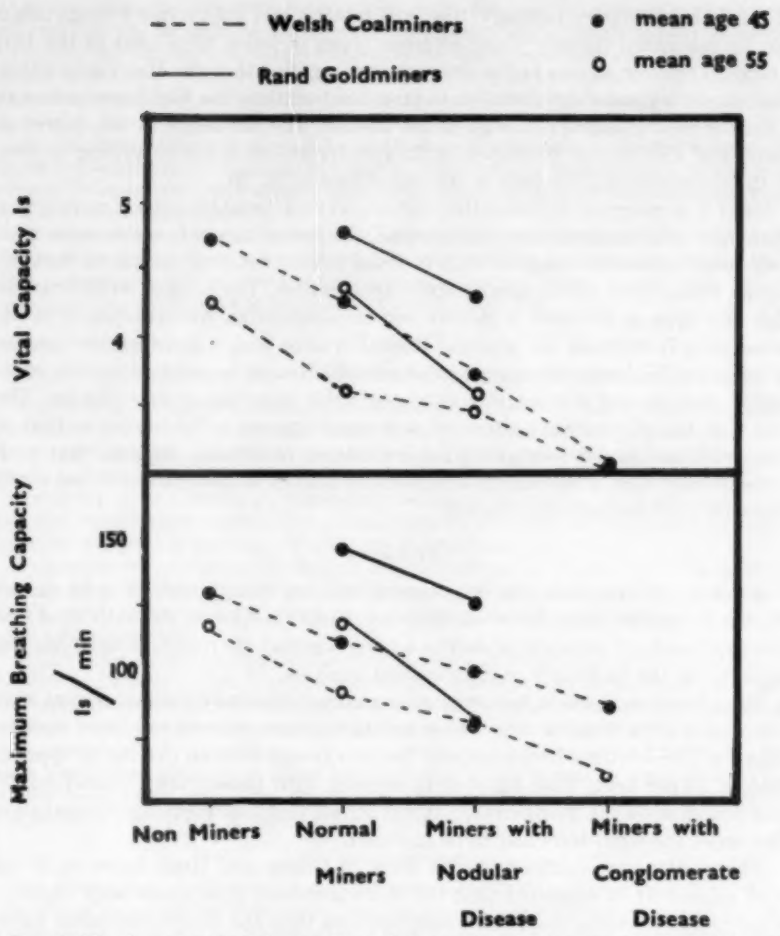

FIG. 2. The decrease of vital capacity and maximal breathing capacity in Welsh coal miners compared with that in Rand gold miners showing similar roentgenographic grades of dust disease.

industries, it was thought that a comparison of the degree of the physiologic disturbances with the different roentgenographic categories of the two types of miners would be of interest. The decrease in vital capacity and maximal breathing capacity in the gold miners is greater than in the coal miners at a similar roentgenographic stage of disease, as maybe seen in figure 2. Absolute values for both vital capacity and maximal breathing capacity are higher in the roentgenographically normal Rand miners than in the Welsh coal miners, a difference which could be due to differences in technique or differences in body size, since the Welshmen were on the whole smaller in size than the South Africans. Differences

in the intrapulmonary mixing of the coal miner's and gold miner's lungs could not be calculated directly since different types of index were used in the two studies. The over-all conclusion thus appears justified that the Rand gold miner may expect a greater deterioration in lung function than the Welsh coal miner at a similar roentgenographic stage of his disease. The difference in the degree of functional disturbance developed in the two conditions is not surprising in view of the difference in pathology of the two diseases (25, 26).

Since it is generally believed that service on rock-breaking jobs is more dusty than most other underground occupations, the correlation of function score with rock-breaking service suggests that in Rand miners function relates to dust exposure irrespective of roentgenographic appearances. The present writers realize that this type of evidence is slender and circumstantial. Nevertheless, it is believed that it supports the growing clinical opinion that a considerable number of miners suffer from pulmonary symptoms which must be ascribed to their occupation, despite the absence of roentgenographic evidence of dust disease. The fact that the physiologic pattern in such cases appears to be similar to that of men with undoubted roentgenographic evidence of silicosis suggests that such patients may have a fair degree of pulmonary fibrosis of a sort which is not easily demonstrated roentgenographically.

Summary

A study of lung function was carried out on Witwatersrand gold miners chosen at random from the whole industry. Lung function in the individual case was expressed as a score calculated by adding together the results of eight selected tests, using the technique of discriminant analysis.

Lung function scores in men with no significant roentgenographic change were contrasted with those of men whose roentgenograms showed advanced nodular silicosis. The score method separated the two groups with an overlap of approximately 15 per cent. This figure is in keeping with those from Switzerland (8) and Scandinavia (5). Furthermore, it was shown that to achieve discrimination of this order the eight tests had to be included.

The results were contrasted with those of Gilson and Hugh-Jones on Welsh coal miners. It is suggested that the Witwatersrand gold miner may expect a more marked deterioration of his lung function than the Welsh coal miner for an equivalent roentgenographic grade of dust disease.

Lung function scores were shown to correlate with length of rock-breaking service, irrespective of the roentgenographic findings. This suggests that disability due to mining may be found in men in whom there is no frank roentgenographic evidence of dust disease.

Sumario

La Función Pulmonar en la Silicosis de los Mineros de Oro del Witwatersrand

En mineros de oro tomados al azar de toda la industria en el Witwatersrand se llevó a cabo un estudio de la función pulmonar. En los distintos casos, ésta se expresó en forma de cómputo obtenido sumando los resultados de ocho pruebas escogidas en que se usó la técnica del análisis discriminante.

Se compararon las cifras de la función pulmonar en hombres sin importantes alteraciones radiográficas con las de hombres cuyas radiografías revelaban silicosis nodular avanzada. El método del cómputo separó a los dos grupos con una sobreposición aproximadamente de 15 por ciento. Esta cifra se conforma a las de Suiza (8) y de Escandinavia (5). Ademas, se observó que para obtener una diferenciación de este orden, había que utilizar las ocho pruebas.

Se compararon los resultados con los de Gilson y Hugh-Jones en mineros de carbón de Gales. Sugiérese que, con un grado radiográfico equivalente de neumoconiosis, el minero de oro del Witwatersrand puede esperar un deterioro más notable de su función pulmonar que el minero de carbón de Gales.

Se demostró que las cifras de la función pulmonar se correlacionaban con el período de tiempo dedicado a triturar rocas, independientemente de los hallazgos roentgenográficos. Esto sugiere que puede encontrarse incapacidad debida al mineraje en sujetos en los que no existen signos radiográficos declarados de neumonoconiosis.

Resume

La fonction pulmonaire chez les mineurs d'or de Witwatersrand atteints de silicose

Une étude de la fonction pulmonaire a été faite chez les mineurs d'or de Witwatersrand—choix fait au hasard dans l'ensemble de cette industrie. La fonction pulmonaire dans chacun des cas est exprimée comme une valeur (score) calculée en additionnant ensemble les resultats de 8 tests sélectionnés et en appliquant la méthode d'analyse discriminative.

Les scores de la fonction pulmonaire des sujets ne présentant pas d'altérations pulmonaires radiologiques significatives furent comparés avec ceux des sujets dont les radiogrammes révélaient la présence d'une silicose nodulaire accentuée.

La méthode des scores séparait les deux groupes avec un chevauchement évalué approximativement à 15%. Ce chiffre est comparable à ceux obtenus en Scandinavie (5) et en Suisse (8). En outre, la nécessité d'effectuer les 8 tests pour réaliser une discrimination de cet ordre a été démontrée.

Les résultats ont été confrontés avec ceux de Gilson et Hugh-Jones chez les mineurs de charbon du Pays de Galles. L'article suggére que le mineur d'or de Witwatersrand peut s'attendre à subir une altération plus marquée de sa fonction pulmonaire que celle que subira le mineur gallois pour un même degré de silicose décelé sur le radiogramme.

Les scores de la fonction respiratoire correspondaient à la longueur du travail dans la mine, sans qu'interviennent les signes radiologiques. Ce fait permet de supposer que l'invalidité résultant du travail de mineur peut être observée chez celui-ci dans des cas où la radiologie ne revèle pas de lésions silicosiques nettes.

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A CONTROLLED INVESTIGATION OF STREPTOMYCIN TREATMENT IN TUBERCULOSIS[1]

A Five-Year Follow-up Study

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Introduction

In October, 1947, four New York State Tuberculosis hospitals, along with a number of other tuberculosis hospitals in the United States, joined a cooperative study to determine the effect of adding streptomycin alone as a therapeutic agent to other therapeutic measures in the treatment of pulmonary tuberculosis. This study was done in cooperation with the United States Public Health Service and the results at the end of one year's observations have been reported by Long and Ferebee (1).

In the original study there were 541 patients, 214 of whom came from the New York State hospitals. The present study is a five-year follow-up of these 214 cases. It was undertaken to determine if the greater improvement achieved by patients treated with streptomycin as compared with untreated control subjects had continued for an extended period or if the early gains were gradually lost as time went by.

Plan of Investigation

A brief description of the original study done in 1947 and a statement of the early results relating to the New York State cases is needed to establish a background for the present report. The method of investigation was as follows: Only patients with moderately advanced and far advanced disease were selected; minimal and terminal cases were excluded. Selection was strictly at random. The only variable was streptomycin therapy, which was given in amounts of 20 mg. per kg. of body weight per day for 91 days to treated patients but not to control subjects. (Streptomycin was the only antituberculosis drug then available.) Any other form of treatment for tuberculosis was allowed for any member of either group. At the end of one year and at all observation points up to that time there was, for every characteristic that was analyzed, a marked and consistent superiority of the streptomycin-treated cases over the untreated controls.

Observations

The New York State cases comprised 107 treated cases and 107 untreated cases. The initial characteristics of the two groups were comparable (table 1). The status of the disease at the end of a one-year observation period called for in the original experimental protocol is presented in table 2. The 1950 *Diagnostic Standards and Classification of Tuberculosis of the National Tuberculosis Association* are used as criteria. Use of the 1955 *Diagnostic Standards* would place those patients designated as arrested into the active category. The reason for this is

[1] From the Mount Morris Tuberculosis Hospital, Mount Morris, New York.

TABLE 1

CHARACTERISTICS OF 107 STREPTOMYCIN-TREATED AND 107 UNTREATED CASES AT TIME OF SELECTION FOR STUDY

New York State Hospitals Only*

Characteristics and Treatment		Streptomycin	Control
Race	White	94.4	96.3
	Non-white	5.6	3.7
Sex	Male	49.5	54.2
	Female	50.5	45.8
Age	Less than 25	29.0	28.0
	25–44	54.2	49.6
	45 and older	16.8	22.4
Length of hospitalization prior to entering study	Less than 3 months	55.2	51.4
	3 to 5 months	16.8	16.8
	6 to 11 months	14.0	15.0
	1 year or more	14.0	16.8
Temperature	Markedly febrile	9.3	9.3
	Slightly febrile	57.0	40.2
	Afebrile	33.7	50.5
Body weight	Normal or overweight	37.1	36.6
	Underweight	62.9	63.4
Amount of sputum	None	37.4	37.7
	Slight	25.2	28.3
	Moderate	28.0	27.4
	Copious	9.4	6.6
Previous streptomycin therapy	Streptomycin	4.7	5.6
	No streptomycin	95.3	94.4
Surgery or collapse procedures in effect†	None	92.5	87.8
	Pneumothorax	4.7	4.7
	Pneumoperitoneum	0.9	2.8
	Thoracoplasty	1.9	4.7
State of disease	Moderately advanced	59.4	64.5
	Far advanced	40.6	35.5
Predominate morphology	Exudative	37.0	40.9
	Caseous	47.5	38.0
	Fibrotic	15.5	21.1
Dynamics	Acute	30.4	28.1
	Subacute	43.1	44.0
	Chronic	26.5	27.9

TABLE 1—*Continued*

Characteristics and Treatment		Streptomycin	Control
Course	Progressive	60.8	52.7
	Stationary	31.7	38.0
	Retrogressive	7.5	9.3
Cavitation	Definitely present	80.1	79.2
	Not present	19.9	20.8

* Hermann M. Biggs, Homer Folks, Mount Morris, and Ray Brook Hospitals.

† Patients receiving more than one form of surgery or collapse procedure are duplicated.

TABLE 2

STATUS AT END OF ONE YEAR

	Inactive	Arrested	Active	Dead	Unknown	Appeals
Treated group (107).....	14	35	56	2	0	—
Control group (107).....	0	18	78	3	5	3

that, although inactive disease has the same definition in the 1955 *Diagnostic Standards* as it did in the 1950 *Diagnostic Standards*, the less rigid criteria of arrested disease in the earlier edition (briefly, absence of symptoms referable to tuberculosis, stable chest roentgenograms without cavity, and sputum negative for *M. tuberculosis*, all for three months) have no exact counterpart in the 1955 *Diagnostic Standards*; inasmuch as they do not fulfill the requirements for inactive status, these patients would now be listed as having active disease.

It will be noted that, by the end of the one-year observation period, 3 patients in the untreated group had already received streptomycin for life-threatening episodes as provided for in the original protocol. These patients are the "appeals" noted in table 1 which were listed as "not available" in the original report. They are excluded from further consideration here except for mention of the fact that, on the fifth anniversary of their entrance to the study, 2 of these patients had inactive tuberculosis and one had arrested disease.

The status of the patients in the two groups on the fifth anniversary of their entrance into the study may be seen in table 3. It is apparent that the status of the originally treated patients is more favorable than that of the untreated group, especially from the standpoint of inactive disease and deaths. All of the 9 treated patients listed as dead died of tuberculosis, while 21 of the 23 dead control subjects died of tuberculosis.

During the last four years of the five-year period covered by this study, additional treatment was given to different subjects according to clinical indications and without protocol restriction. This is summarized in table 4. With regard to collapse and excisional therapy, only one procedure (the one thought to be most definitive) is listed for each individual, although several of the patients had more than one such form of treatment. The type and duration of drug therapy varied

TABLE 3

STATUS AT END OF FIVE YEARS

	Inactive	Arrested	Active	Dead	Unknown
Treated group (107)	55	26	14	9	3
Control group (104)	38	17	18	23	8

TABLE 4

ADDITIONAL THERAPY

	Drugs	Pneumothorax or Pneumoperitoneum	Thoracoplasty	Excision	Unknown
Treated group (107)	41	15	30	8	1
Control group (104)	59	17	21	15	5

TABLE 5

ADDITIONAL THERAPY IN PATIENTS WITH ARRESTED OR INACTIVE DISEASE

	Drugs Only	Collapse or Excision Only	Drugs plus Collapse or Excision	No Drugs, Collapse, or Excision
Treated group (81)	4	27	20	30
Control group (55)	7	16	25	7

so greatly as not to be susceptible to analysis. The 41 patients of the treated group had 14,065 patient-days of treatment after the original 91 days of streptomycin therapy; and the 59 control subjects subsequently had 13,265 patient-days of treatment. The average duration of this treatment was, therefore, 343 days for the treated group and 225 days for the control group. The median duration of treatment in both the treated and control groups was 275 and 183 days, respectively. Various clinical indications were evidently responsible for the difference. The same can be said of collapse or excisional therapy; the total number thus treated in each group was the same.

At the fifth anniversary of entry to the study, a disease status of inactive or arrested had been achieved by 81 of the 107 patients in the treated group and by 55 of the 104 in the control group. The combinations of treatment that were used to bring about this result is shown in table 5. The original treatment with streptomycin is excluded. It is noted that there is little difference between the two groups except in the numbers who were thought to require no additional treatment with drugs or other therapy. Thirty of the treated group and only 7 of the control group were thought to require no further treatment.

SUMMARY

A follow-up study of 214 patients with moderately or far advanced pulmonary tuberculosis has been presented. Half of the patients received streptomycin daily for the first ninety-one days as their only drug treatment during the first

year, and half received no antituberculous chemotherapy during the first year. After that, chemotherapy was available to all. The originally treated patients retained the advantages gained during the first year, as shown by analysis of their status on the fifth anniversary of entrance to the study. This is believed to demonstrate the seldom-proved proposition that chemotherapy, delayed for one year, will not yield as good results as it would if given promptly.

Sumario

Investigación Fiscalizada de la Estreptomicinoterapia en la Tuberculosis: Cinco Años de Observación

Se presenta un estudio de observación de 214 enfermos que tenían tuberculosis pulmonar moderada o muy avanzada. La mitad de los enfermos recibió estreptomicina diariamente durante los primeros noventa y un días como único tratamiento medicamentoso el primer año. Después de esto, se puso la quimioterapia a la disposición de todos. Los enfermos tratados primitivamente retuvieron las ventajas obtenidas durante el primer año, según demuestra el análisis de su estado en el quinto aniversario de haber entrado a formar parte del estudio. Esto parece apoyar la proposición rara vez probada de que la quimioterapia, demorada un año, no dará tan buenos resultados como los que daría, si se administrara prontamente.

Resume

Une investigation contrôlée de la Streptomycine dans le traitement de la tuberculose: Une période d'observation de cinq ans

Présentation d'une étude comportant 214 tuberculeux avec lésions pulmonaires modérées ou avancées qui furent surveillés pendant cinq ans. La moitié des cas reçut uniquement la streptomycine chaque jour pendant les quatre-vingt-onze premiers jours de la première année du traitement et l'autre moitié ne reçut aucune chimiothérapie antituberculeuse pendant la première année. La chimiothérapie fut ensuite appliquée à tous les malades. Les malades traités dès la première année conservèrent les avantages gagnés au cours de la première année, comme le démontre l'analyse de leur état qui est effectuée au cinquième anniversaire de leur inclusion dans l'étude. Ce fait paraît corroborer une conception rarement démontrée, à savoir qu'un délai d'un an dans l'application de la chimiothérapie donne de moins bons résultats que lorsque celle-ci est pratiquée précocement.

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TUBERCULOSIS IN THE NEONATAL PERIOD[1, 2]

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INTRODUCTION

While it is categorically considered necessary to remove newborn infants from contact with tuberculous mothers whose pulmonary disease is in an active state, two important questions remain unanswered: (*1*) Should infants born of mothers with inactive tuberculosis be removed from contact with them if the tuberculous disease in the mother has recently been considered active? (*2*) What should constitute the proper diagnostic and therapeutic approach to an infant who is demonstrated to have been exposed to active tuberculosis in its mother?

The ensuing follow-up study of 60 infants born of tuberculous mothers attempts to bring these problems into focus.

OBSERVATIONS

Sixty infants (table 1), born of mothers who had active pulmonary tuberculosis at the time of delivery, or recently had active pulmonary tuberculosis, or who had contracted pulmonary tuberculosis while the child was in early infancy, constitute the study. Nine of the infants received BCG vaccine. Twenty-one of the remaining 51 infants were infected with tuberculosis and 3 of these developed tuberculous meningitis. Death resulted in one of these cases (*E. H.*) (1). One other patient in the series (*E. C.*) died, but this patient, who was separated from his mother at birth, had apparently received poor general care. The patient was hospitalized with diarrhea shortly after birth and died at the age of one month. At autopsy the diagnosis was pneumonia and inanition, with no evidence of tuberculosis.

Type of Tuberculous Infection

Of the 21 infants infected with tuberculosis (table 2), 13 had primary pulmonary tuberculosis demonstrable by roentgenography. Another infant (*E. H.*), who had pulmonary tuberculosis at the age of thirty days, developed tuberculous meningitis and died seven months after the onset of the infection. Two other patients with tuberculous meningitis survived. There were 2 infants with tuberculous pneumonia. One of these (*L. K.*), whose mother had a known case of miliary tuberculosis, was inadvertently put to breast at one time during the first twenty-four hours of life and became infected at the age of twenty-three days (1). The patient subsequently developed tuberculous pneumonia, but

[1] From the Department of Pediatrics and the Child Chest Clinic, Medical College of Virginia, Richmond, Virginia.

[2] This study was aided by a grant from the A. D. Williams Memorial Fund as of June 1, 1957.

TABLE 1

INFANTS BORN OF TUBERCULOUS MOTHERS

Infected	21
Noninfected	30
BCG-vaccinated	9
Total	60

TABLE 2

PREDOMINANT TYPE OF TUBERCULOUS INFECTION AMONG INFANTS

Tuberculous Disease	Cases
Primary pulmonary	13
Meningitis (1 with pneumonia)	3
Positive Mantoux reaction (negative roentgenogram)	3
Pneumonia	1
Pulmonary	1
Total	21

made an uneventful recovery on therapy with isoniazid (thirteen months) and streptomycin (three months). Another patient (*R. L. K.*) was born of a mother who had tuberculosis with sputum positive for tubercle bacilli, and remained in the same room with her for ten to twelve hours following the birth (2). The mother and child were brought to the hospital in the same ambulance. On the nineteenth day of life, the infant had a positive Mantoux reaction, and a roentgenogram revealed widespread pulmonary tuberculosis. Among those 3 infants who were tuberculin positive but exhibited no other evidence of disease, there was one whose infection was demonstrated at the age of eleven weeks.

Contact with the Mother

As previously mentioned, 9 of the infants received BCG vaccine. Only 3 of the remaining 51 infants had no contact with their respective mothers. One of these 3 (*E. C.*) died of pneumonia and inanition at the age of one month, after a previous hospitalization because of diarrhea. There was no evidence of tuberculosis at autopsy. The other 2 (*D. W.* and *E. K.*) have had no contact with their mothers and are still tuberculin negative.

The other 48 patients had at least minimal contact with their mothers, and 21 of them became infected with tuberculosis (table 3). It is noteworthy that in only 7 of these cases was the mother found to be discharging tubercle bacilli in the sputum (table 4). The remaining 14 mothers of infected children were considered noninfectious, and an examination of household contacts revealed no other source of tuberculous disease. The majority of the infected infants had been in close contact with their mothers, but this group does include the offspring of 4 mothers who were found to have active pulmonary tuberculosis

TABLE 3

CONTACT WITH TUBERCULOUS MOTHERS

	No Contact	Contact
Infected	0	21
Noninfected	3	27
Total	3	48

TABLE 4

KNOWN INFECTIVITY OF TUBERCULOUS MOTHERS OF INFECTED CHILDREN

Sputum positive for *M. tuberculosis*	7
Sputum negative for *M. tuberculosis*	14
Total	21

TABLE 5

INFANTS NOT INFECTED WITH TUBERCULOSIS

No contact with mother	3
Constant contact with mother	23
Separated at birth for various periods	4
Total	30

after the infant was born. In 3 instances this diagnosis was made when the baby was only a few weeks old. From the 4 mothers one case of tuberculous meningitis (*L. M.*) resulted, one of tuberculous pneumonia (*R. F.*), and two cases of primary pulmonary tuberculosis (*C. S.* and *W. H.*). The brief contact with their offspring during the first day of life of 2 known tuberculous mothers who had sputum positive for tubercle bacilli resulted in widespread pulmonary tuberculosis in the 19-day-old infant (*R. L. K.*) of one mother and tuberculous pneumonia in the other infant (*L. K.*), who was twenty-three days old.

In 6 instances, the tuberculous mother was separated at birth from the infant for periods varying from seven weeks to two years, and each mother had sputum negative for tubercle bacilli when finally permitted to assume care of the child. One of these mothers, however, had made regular visits to her baby (*L. W.*) in a foster home previous to assuming such care. The remaining 9 mothers, who were considered noninfectious, were in constant contact with their offspring except for one mother who had a relapse of her disease and entered the sanatorium for further treatment.

Of the group of 30 infants not infected with tuberculosis (table 5) 4 were separated from their mothers at birth for periods ranging from three months to two years. The other 23 were in constant contact with their mothers, 21 of these mothers being considered noninfectious at the time although some still received antimicrobial therapy and 3 required further sanatorium care.

Two other infants escaped infection in spite of intimate exposure to their

mothers, who were found to have active pulmonary tuberculosis when the infants were four and nine months of age, respectively. The former infant (*A.B.*) was breast fed and the latter (*C.C.*) slept with his mother.

Discussion

From the material at hand, it appears that contact of an infant with a mother who has supposedly inactive tuberculous disease and sputum negative for tubercle bacilli may not be as safe as expected. Nine of the 30 infants in this category became infected with tuberculosis, and one died with tuberculous meningitis. Proof is not available to show that these infants were infected by the mother; however, one of the 8 mothers was forced to return to the sanatorium for further treatment.

It cannot be said that temporary separation of an infant from a mother with active tuberculous disease is always successful in preventing infection of the child. Of the 10 infants who were separated from their mothers for periods ranging from seven weeks to two years, or until the mother had noninfectious sputum and appeared to have arrested disease, 6 developed tuberculous infection. None of these infants, however, suffered serious tuberculous disease.

There was no case of clinical tuberculosis among the 9 patients in this series who received BCG vaccine. Six of them were immediately separated from the mother, however, and only 3 were kept in close contact. Although no conclusion can be drawn from this small group, Rosenthal (3) has reported that among infants born of tuberculous parents, those who receive BCG vaccine have a more favorable prognosis than those who do not have the benefit of this prophylactic approach.

Grady and Zuelzer (4) have suggested that infants born of mothers who have active pulmonary tuberculosis be given prompt antimicrobial therapy. The effectiveness of such an approach is questionable, for Schmidt's work on simians (5) has demonstrated that the institution of chemotherapy at the inception of infection may result only in prolongation of the incubation period. Fifty per cent of his rhesus monkeys developed tuberculosis when the antimicrobial therapy was discontinued.

It would appear, therefore, that the best approach is: careful observation of the newborn infant, with an immediate chest roentgenogram and tuberculin test; isolation from the mother for a period of six weeks; and BCG vaccination and isolation thereafter for a minimum of six weeks, or until the mother's tuberculous disease is so controlled that contact with the infant is advisable.

The effectiveness of an approach involving *only* careful observation is exemplified in a group where there were 5 infants infected with tuberculosis at eleven weeks of age or less. One baby had widespread pulmonary tuberculosis at nineteen days, 2 developed tuberculous pneumonia at twenty-three and thirty days, respectively, and one was found to have primary pulmonary tuberculosis at five weeks of age. Another infant developed a positive Mantoux reaction at eleven weeks of age. Only one of these patients died and this child, who was infected at thirty days of age, had later developed tuberculous meningitis in the era before the advent of isoniazid.

Summary

Nine of the 30 infants born of mothers with supposedly inactive tuberculosis became infected with the disease. Of the 10 infants who were separated at birth from their mothers for periods ranging from seven weeks to two years, 6 developed tuberculous infection. This study suggests that isolation of infants until the mother's sputum is negative for tubercle bacilli and she is considered to have inactive disease may not be enough to prevent infection in the infant. Supportive evidence is not presented here but, on the basis of studies by others with BCG, it is suggested that temporary isolation and BCG vaccination appear to constitute the best approach to the problem.

Sumario

Tuberculosis en el Período Neonatal

Nueve de los 30 lactantes dados a luz por madres que tenían tuberculosis supuestamente inactiva se infectaron con la enfermedad. De los 10 lactantes que fueron separados al nacer de las madres por un período que varió de siete semanas a dos años, 6 manifestaron infección tuberculosa. Sugiere este estudio que el aislamiento de las criaturas hasta que el esputo maternal sea negativo para bacilos tuberculosos y se considere que la madre tiene enfermedad inactiva quizás no baste para impedir la infección del lactante. No se presentan aquí datos corroboradores, pero a base de la labor de otros autores, se señala que el aislamiento temporal y la vacunación con BCG parecen constituir el mejor aborde al problema.

Resume

La tuberculose dans la période néo-natale

Parmi 30 enfants nés de mère atteintes d'une tuberculose considérée stabilisée, 9 d'entre eux devinrent tuberculeux. Parmi 10 nourrissons qui, à la naissance, furent séparés de leur mère pendant une période variant entre sept semaines à deux années, 6 devinrent tuberculeux. Cette étude suggère que l'isolement des enfants jusqu'au moment où l'expectoration de la mère devient "négative" peut n'être pas une mesure suffisante pour prévenir l'infection chez l'enfant. Les données confirmant ces vues ne sont pas exposées dans l'article, mais, en se fondant sur les travaux d'autres auteurs, l'auteur suggère que l'isolement temporaire et la vaccination par le BCG paraissent devoir constituer le meilleur moyen d'aborder le problème.

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A STUDY OF THE GROWTH IN HELA CELLS OF TUBERCLE BACILLI FROM HUMAN SPUTUM[1]

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Introduction

It has been reported previously that tubercle bacilli and certain other mycobacteria of pathogenic potential grow rapidly when introduced into HeLa cells (1–3). In those studies the mycobacterial inocula for the cells were prepared from young cultures growing in Tween®-albumin medium. It was of interest to learn the comparative activity of mycobacteria as they are found in diseased tissue, and the present study concerns tubercle bacilli present in human sputum. The general working hypothesis was that in a chronic infectious disease such as tuberculosis the condition of the infecting agent should be of critical importance. The studies of Medlar and associates (4) have shown that the culturability of tubercle bacilli varies markedly in different types of pulmonary tuberculous lesions. It seemed possible that the ability of tubercle bacilli to grow in human cells in tissue culture would bear more prognostic significance than their culturability on bacteriologic medium.

The experimental approach was to inoculate sputum concentrates from individual patients into several tubes of HeLa cell cultures so that preparations could be removed for study at appropriate intervals. Many of the tubercle bacilli in the sputum proved viable and grew rapidly. Because they grew in well-defined intracellular groups, it was possible to estimate the percentage of viable organisms in the sputum samples and to measure approximately the rate of growth of the bacilli in their first few generations in HeLa cells. Thus some idea could be gained of the individual behavior of the bacilli from diseased human tissue as they began to grow in the HeLa cells. The high level of viability observed for the tubercle bacilli in sputum provides a background of experience for the evaluation of a study of resected pulmonary specimens that is in progress.

The possibilities of HeLa cell cultures in the laboratory diagnosis of tuberculosis were studied by comparing the results seen in the tissue cultures with those obtained by also plating out each concentrate on a bacteriologic medium in frequent use in diagnostic laboratories.

Materials and Methods

The methods employed for the HeLa cells have been described in detail elsewhere (2). In brief, they were as follows: HeLa cells were grown on coverslips in Leighton tubes for two or three days in 40 per cent human serum and 60 per cent Hanks' balanced salt solution (BSS, (5)). The cells were then washed on their coverslips in BSS, and 1 ml. was added of an "infection medium" (10 per cent of a selected horse serum in BSS containing the amino acids and vitamins recommended by Eagle (6)). The sputum concentrates were

[1] From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Montgomery, Alabama.

then inoculated in 0.05 ml. amounts. On the following day the medium was changed back to the growth medium, and further changes of tissue culture fluid were made every two days. Penicillin (100 units per ml.) was present in all cultures which had received sputum. Coverslip preparations were washed and fixed for staining usually one, three, five, and seven days after addition of the sputum concentrates. Each sputum was digested with an equal volume of 4 per cent sodium hydroxide for twenty minutes at room temperature. Shaking was done by hand every few minutes with special attention to the less readily digested sputum aggregates. The sodium hydroxide was then neutralized. With the last 20 sputum specimens, this was done by drop-wise addition of 1 N HCl in BSS until the color changed to yellow, followed by drop-wise addition of N/10 sodium hydroxide in BSS until an orange color resulted. The sputum specimens were then centrifuged at 3,000 r.p.m. for thirty minutes, the supernatants discarded, and the sediments resuspended in 2 ml. of BSS by forceful expulsion with bulb and pipette against the bottom of the tube. There was no indication that the sodium hydroxide failed to kill all other organisms beside tubercle bacilli because the only contaminants observed were infrequent molds growing inward from the side of the plate and from outside the inoculated area. None of the tissue cultures receiving these concentrates showed contamination.

The 25 per cent blood agar of Tarshis and associates (7) in Petri dishes was inoculated in 0.05 ml. amounts of the undiluted material as well as dilutions of 10^{-3} and 10^{-4} in BSS. The colonies were counted at weekly intervals until a maximum was reached.

Smears of undiluted concentrates were stained and examined microscopically. Usually the sputum specimens were selected for their high content of bacilli so that the number of infected cells would be great enough for convenient study.

Microscopic examination of stained HeLa cells on their coverslips always included enumeration of the bacilli with a 97× oil immersion lens over a representative area of known dimensions. The coverslips measured 8 by 30 mm., and the diameter of the microscopic field was 0.21 mm. The size of the area examined depended on the number of bacilli present and was either several standard representative microscopic fields or one or more lengthwise sweeps of the coverslip. Each microscopic field represented $\frac{1}{7,000}$th part of the coverslip, and each lengthwise sweep $\frac{1}{58}$th part. From the area examined the total number of organisms in the cells on the coverslip could be estimated. The sputum concentrates contained voluminous particulate matter that tended to aggregate, making it difficult to provide each tube with equivalent inocula. This factor is presumably the greatest source of variation in total counts between tubes.

Aliquots of some sputum samples were also concentrated after trypsin digestion. One to 2 ml. of sputum were mixed with 5 ml. of a 0.25 per cent solution of the enzyme (Difco 1:250) in salt solution containing the NaCl, KCl, and glucose of BSS (8) at pH 7.5. Incubation at 37°C. usually did not exceed a total of fifteen minutes since, after five minutes of incubation, the sputum could be broken up by aspiration and expulsion with bulb and pipette. A second five-minute incubation followed by another treatment with bulb and pipette was sufficient to eliminate viscous material and allow concentration of bacilli by centrifugation. The sediment was resuspended in 1 ml. of BSS.

This treatment did not eliminate other bacteria. Smears and cultures revealed a high concentration of the usual organisms of the respiratory tract. Fortunately these did not usually grow in the tissue culture media. Many of the cells in the sputum were also concentrated by this procedure, and occasionally tubercle bacilli and other organisms were seen to have been engulfed by leukocytes. This procedure was selected for its mildness, and it was a modification of that found by Puck and associates to give well-dispersed suspensions of HeLa cells, nearly 100 per cent of which were capable of growth from single cells (8).

Although a total of 116 sputum samples have been studied, this report is limited to the 61 specimens that were treated according to the technique described. With these, there were 45 instances in which tubercle bacilli were found in the HeLa cells. The incidence of positive findings by itself does not reflect the sensitivity of the method since the sputum

specimens were selected for high content of bacilli. The specimens reported were received through the courtesy of Dr. Robert K. Oliver of the Montgomery Tuberculosis Sanatorium. Some of the sodium hydroxide concentrations were performed through the cooperation of Dr. Thomas S. Hosty of the Bureau of Laboratories of the Department of Public Health of Alabama. The last 20 specimens were treated with care to prevent overneutralization with sodium hydroxide, and the cells into which they were inoculated were probably in a better condition due to several minor improvements in the care and nutrition of the cells.

Results

With the techniques employed, infection of the HeLa cells was limited primarily to the first day after inoculation, since phagocytosis of tubercle bacilli by these cells is very much greater in "infection medium" than it is in the growth medium (1). The subsequent results thus largely represented the developments of bacilli resident in the cells since the day of inoculation.

A typical result on the first day following inoculation is shown in figure 1. Clumping of bacilli from sputum was very rarely encountered, and usually groups of one, two, or three bacilli were observed at this stage. At three days (two days later) evidence of growth was seen in an increase in the size of the bacterial groups (figure 2). At five days growth was more extensive (figure 3), and frequently at seven days growth of bacilli had broken out of the cells, so that extracellular bacilli were often seen (figure 4).

Thus the principal events with tubercle bacilli from sputum did not appear very different from those with bacilli from bacteriologic medium (2). This result was not expected because it is commonly accepted that the sodium hydroxide procedure is harmful to tubercle bacilli, as witnessed by statements to this effect in the standard textbooks. Furthermore, it had been thought that microorganisms from a disease as chronic as tuberculosis might show evidence of exposure to resistive forces of the body when first removed from their environment in diseased tissue. To gain more definite information on the physiologic condition of the tubercle bacilli in sputum, the following studies were carried out concerning their viability rates and generation times.

"*Viability rates*": A differential count of groups of tubercle bacilli according to the number of bacilli per group was made on coverslip preparations removed on succeeding days. The size of 50 groups was usually recorded for each coverslip. A summary of the distribution by size of bacterial groups for three specimens inoculated on the same day is given in the last five columns of table 1. There was a steady increase in the average size of bacillary groups with each two-day interval. This increase in the average size of the groups is thought to represent bacterial multiplication, because: it occurred with characteristic cording and parallel alignment; phagocytosis of mycobacteria by HeLa cells after the first day would be at a minimum in the growth medium; and there was no tendency for progressive change in the total number of bacterial groups on succeeding days. If the percentage of groups containing more than 5 bacilli on the fifth or seventh day is taken as an estimate of the "viability rate," figures of 72, 70, and 70 per cent are obtained for the three sputum specimens in table 1. The same estimate was made for the 37 specimens containing enough tubercle

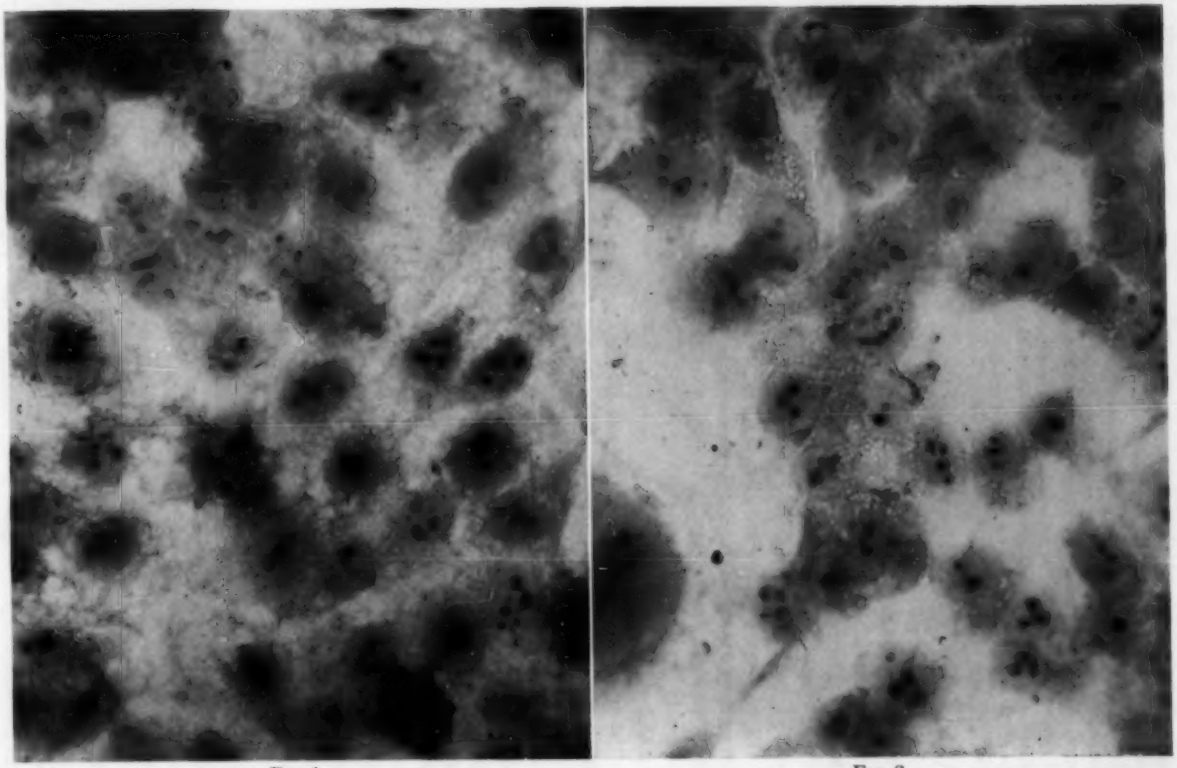

FIG. 1. FIG. 2.

FIG. 1 (Left). One day after addition of a concentrate of tuberculous sputum to HeLa cells. The individual tubercle bacilli have been phagocytosed but have not yet begun to grow. (× 785)

FIG. 2 (Right). At three days (two days later) some of the bacilli have multiplied to form small groups. (× 785)

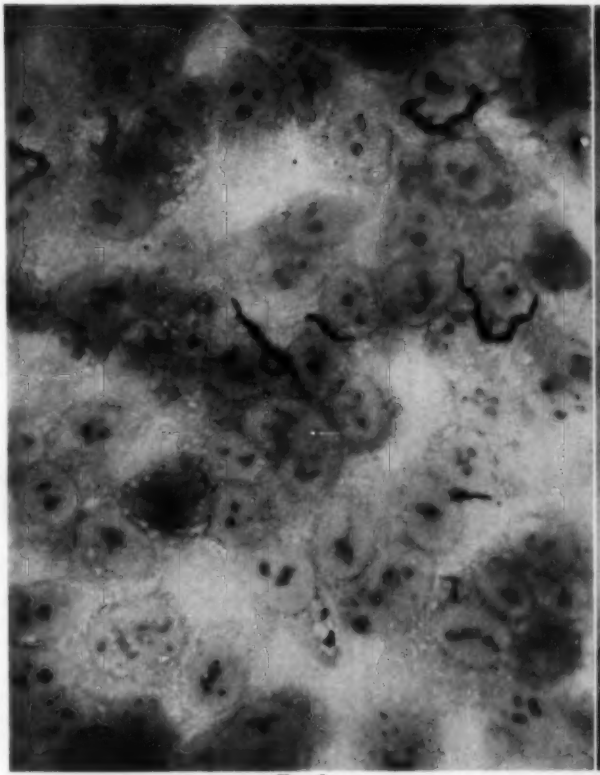

FIG. 3.

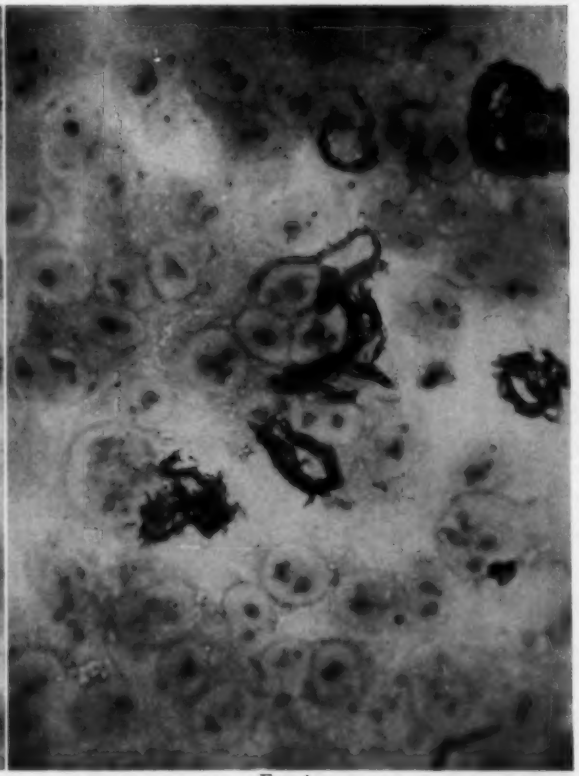

FIG. 4.

FIG. 3 (Left). At five days the intracellular growth of the tubercle bacilli is manifested by the formation of distinct cords of bacilli. (× 785)

FIG. 4. (Right) At seven days growth of bacilli has progressed even more and in other microscopic fields had broken out of the cells. Note the characteristic perinuclear position of the cords of bacilli. (× 785)

bacilli to allow a differential count on the seventh day (or fifth day if the seventh day preparation was unsatisfactory). The average figure for the 37 sputum specimens was 54 per cent (range 12 to 89 per cent).

The last 20 sputum specimens studied were treated with greater care to prevent overneutralization with HCl and with greater attention to the physiologic state of the HeLa cells. The average "viability rate" for the eight of these containing sufficient bacilli was 74 per cent (range 62 to 87 per cent). When strain H37Rv was grown six days in Tween-albumin medium (Difco's Tb-Tween) a "viability rate" in HeLa cells of about 80 per cent was observed (9). Thus it seems clear that, when treated with care, most of the tubercle bacilli in the sputum were capable of growth in HeLa cells even after treatment with 2 per cent sodium hydroxide.

It is possible that the high "viability rate" could also arise because the cells phagocytosed living tubercle bacilli much more actively than dead bacilli. This possibility was investigated by comparing the results with heated (56° C. for sixty minutes) sputum concentrates to those with unheated aliquots of the same samples and mixtures of equal parts of the heated and unheated aliquots. Four sputum concentrates were treated in this manner and no significant differences due to heating were observed.

Generation time: When the differential counts were made, the estimated number of bacilli was recorded for each group. An estimate of the generation time could then be made in the following manner: The total number of bacilli in the 50 groups counted was obtained for each day of observation (table 2, column 3). The generation time represented by the increase in the preceding two days was then calculated (table 2, column 4).

TABLE 1

TOTAL COUNTS AND DIFFERENTIAL COUNTS ACCORDING TO SIZE OF GROUPS OF TUBERCLE BACILLI IN HeLa CELLS AT STATED INTERVALS AFTER INOCULATION WITH CONCENTRATES (NaOH) OF THREE DIFFERENT SPUTUM SPECIMENS

Sputum	Days After Inoculation	Total Bacillary Groups on Coverslip	Size of Bacillary Group				
			1–5	6–10	11–20	21–40	>40
A	1	1.2×10^5	96	4	0	0	0
	3	9.5×10^4	66	24	8	2	0
	5	7.1×10^4	28	20	20	24	8
	7	Too many extracellular bacilli					
B	1	1.5×10^5	98	2	0	0	0
	3	5.8×10^4	96	2	2	0	0
	5	6.6×10^4	54	18	18	8	2
	7	6.6×10^4	30	6	26	14	24
C	1	1.5×10^3	98	2	0	0	0
	3	2.4×10^3	96	4	0	0	0
	5	3.1×10^3	52	14	24	10	0
	7	3.7×10^3	30	6	22	24	18

TABLE 2

METHOD USED FOR THE ESTIMATION OF GENERATION TIME AND "SENSITIVITY RATIO" (LIVE COUNT IN HeLa/PLATE COUNT) FOR THE THREE SPUTUM CONCENTRATES OF TABLE 1

Sputum	Days After Inoculation	Total Bacilli in 30 Groups	Generation Time (days) in Previous 2 Days	Live Count in HeLa	Plate Count	Live Count in HeLa / Plate Count
						per cent
A	1	108	—	—		
	3	248	1.7	—	5.3×10^5	9.7
	5	966	1.0	5.1×10^4		
	7	Too many extracellular bacilli				
B	1	51	—	—		
	3	112	1.8	—	9.4×10^5	4.9
	5	478	0.95	—		
	7	1,214	1.5	4.6×10^4		
C	1	64	—	—		
	3	113	2.5	—	1.8×10^4	14.7
	5	448	1.0	—		
	7	1,264	1.3	2.6×10^3		

The estimated generation times were about two days between the first and third days and about one day between the third and fifth days. Estimates after the fifth day were not reliable because of cellular death and entry of bacilli into the extracellular fluid and because estimates of the number of bacilli in groups containing more than 40 were inaccurate. The last 20 specimens studied provided a few more estimates of generation time in addition to those in table 2. Two more estimates for the interval between the first and third days were 1.8 and 1.3 days and brought the average to 1.8 days; three more for the period between the third and fifth days were 1.0, 0.95, and 0.72 days, so that the average was 1.0 days for this interval. Other estimates were not possible due to the unavailability of one of the preparations defining the two-day interval. The generation time of strain H37Rv in HeLa cells has been estimated to be 1.1 days for both the one-to-three-day period and the three-to-five-day period (10). The bacilli of strain H37Rv used to inoculate the HeLa cells in this instance were grown in Tween-albumin medium. Thus the bacilli from human sputum exhibited a lag phase of about one day when they began to grow in HeLa cells.

Sputum concentrated after trypsin digestion at neutral pH: The last eight sputum samples were also studied after concentration following trypsin digestion. The samples were divided, an aliquot of each was treated by the trypsin procedure, and the remaining portion, by sodium hydroxide. Concentrates by the trypsin procedure contained more nonacid-fast bacteria than they did tubercle bacilli, but little difficulty was occasioned thereby. The nonacid-fast bacteria did not usually grow in the tissue cultures, presumably because of the penicillin present. Phagocytosis of the nonacid-fast bacteria by HeLa cells has been seen only rarely (9) and was not observed with the sputum concentrates. Of the eight sputum specimens studied, six provided results which allowed comparison

between the trypsin and sodium hydroxide concentration procedures. The total counts were lower with most of the trypsin concentrates, in line with the lesser amount of sputum digested by this procedure (1 to 2 ml.). The "viability rates" and generation times were approximately the same for the two methods, however, so that no evidence was obtained that the trypsin treatment was less harmful than the sodium hydroxide.

Susceptibility of irradiated cells: Puck has reported that X-irradiation of HeLa cells with 1,000 r results in the production of giant cells which are incapable of reproduction (11). The use of X-irradiation has also been shown to increase the susceptibility of animals to a number of infectious agents, although the susceptibility of experimental animals to tubercle bacilli has been variously reported to be increased, unaltered, and decreased by this procedure (12). HeLa cells which two days before had received 1,000 r of X-irradiation (250 kvp, 30 ma, filter 0.5 Cu, output 124 r/min. at 50 cm.) were inoculated with sodium hydroxide concentrates of tuberculous sputum. Aliquots of the concentrates were also inoculated into nonirradiated cells. Four specimens of sputum were studied. Differential counts according to the size of the bacillary group showed the "viability rate" and generation time to be approximately the same in irradiated cells as in nonirradiated cells. There was thus no evidence of altered resistance of HeLa cells to tubercle bacilli as a result of irradiation. There was a reduction in the total number of bacilli in the irradiated preparations which appeared to be due to the loss of HeLa cells into the tissue culture fluid.

More extensive studies of the effect of irradiation in even larger doses and with additional tissue culture systems have failed to reveal any increased susceptibility to several species of cultured mycobacteria (10).

Sensitivity of HeLa cell cultures as compared with bacteriologic medium: In order to measure the sensitivity of HeLa cell cultures for the detection of tubercle bacilli in sputum, the tissue cultures were compared with 25 per cent blood agar. First a "live count in HeLa" was defined as the number of bacillary groups containing more than five organisms on the seventh day, or on the fifth day if the seventh-day preparation was not available. The "live count in HeLa" was then divided by its analogous value for bacteriologic media, which was simply the plate count. The result of this division was termed the "sensitivity ratio," since it expressed the number of tubercle bacilli in the concentrate which were grown in HeLa cells compared to the number which could be grown on 25 per cent blood agar.

Calculations for three specimens of sputum are shown in the last three columns of table 2. An analysis of the results for the 61 specimens included in the study is shown in table 3. It will be noted that the most frequent "sensitivity ratio" was in the range of 1 to 5 per cent. There was a large variation between samples, the origin of which must lie in variations in the proportion of bacilli phagocytosed, since it was not associated with variations in the "viability rate." When carefully prepared suspensions of strain H37Rv from Tween-albumin medium were studied, "sensitivity ratios" in the range of 10 to 20 per cent were observed, depending on the dilution of the suspension (9). It appears, therefore,

TABLE 3

COMPARISON OF SENSITIVITY OF HeLa CELL CULTURES AND BACTERIOLOGIC MEDIUM IN GROWING TUBERCLE BACILLI FROM SPUTUM CONCENTRATES

Category	Number of Instances
A. Live count in HeLa = 0 and plate count = 0	13
B. Live count in HeLa = 0 and plate count positive; plate counts ranged from 5 to 630.	8
C. Live count in HeLa positive and plate count positive. The distribution of "sensitivity ratio" (live count in HeLa/plate count) was as follows:	40
1) 0.04 to 0.9% 5	
2) 1.0 to 4.9% 20	
3) 5.0 to 9.9% 7	
4) 10.0 to 19.9% 4	
5) 19.9 to 39.9% 3	
6) 1,900%* 1	
D. Total	61

* Presumably because patient was taking PAS. Bacterial growth was inhibited on the plate receiving 10^{-2} dilution and much more so on that receiving undiluted concentrate.

TABLE 4

THE FRACTION OF THE GROUP OF SPUTUM SPECIMENS FROM WHICH TUBERCLE BACILLI WERE GROWN IN HeLa CELLS (LIVE COUNT IN HeLa > 0) ARRANGED ACCORDING TO PLATE COUNT

	0	1–9	1–9.9 $\times 10^1$	1–9.9 $\times 10^2$	1–9.9 $\times 10^3$	1–9.9 $\times 10^4$	1–9.9 $\times 10^5$	1–9.9 $\times 10^6$
Positive in HeLa	0	0	1	1	7	14	16	1
Total specimens of sputum	13	1	5	4	7	14	16	1

that phagocytosis of tubercle bacilli from sputum concentrates was usually inadequate. Examination of the concentrates leads to the conclusion that it is the very volume of solid material present which interferes with more efficient extraction of tubercle bacilli.

Another analysis of the data was made by arranging the "sensitivity ratios" according to the plate count. The "sensitivity ratio" did not appear to vary unexpectedly with the plate count; consequently, the information could be expressed more simply in the form of table 4, in which it may be seen that, when the plate count was greater than 1×10^3, living tubercle bacilli were always detected in HeLa cells. This figure probably represents a product of the minimal area of the coverslip examined (one lengthwise sweep or 1/38th part of the coverslip), and the most frequent "sensitivity ratio" (about 3 per cent, *C2* in table 3) ($1/38 \times 1/33$ equals 1/1254).

DISCUSSION

The tubercle bacilli here inoculated into HeLa cells were derived from diseased pulmonary tissue, and presumably from open, active lesions. Open cavities

are reported to yield uniformly positive cultures, a finding which puts the proportion of viable bacilli at greater than zero. The present results indicate that at least 50 to 70 per cent of the tubercle bacilli excreted from open lesions may be viable. The percentage of viability and the rate of growth were similar to those observed when the inoculum was prepared from young cultures in bacteriologic medium. Yet it is known that tubercle bacilli in sputum are chemically different from cultured organisms because of their staining with lipid stain (13). It has also been shown that tubercle bacilli grown in the lungs of experimentally infected mice show a greatly decreased response to many added substrates (14).

Although the cytoplasm of the HeLa cell provides an environment for the rapid growth of virulent tubercle bacilli and several other species, this location is considerably different from bacteriologic medium, since it exhibits at least some of the attributes of the intact human. The saprophytes *M. phlei* and *M. smegmatis* do not grow at all in these cells (3), and strains of tubercle bacilli of reduced pathogenicity, such as H37Ra and BCG, grow only slowly (2). The lag phase of about one day sets an upper limit of one day for any period of adaptation required by tubercle bacilli for life in these cells of human origin.

The estimated generation time of tubercle bacilli from sputum when inoculated into HeLa cells is only a little longer than that reported for laboratory strains growing in good bacteriologic medium in the logarithmic phase (15). The small difference that exists may be ascribable to the fact that the organisms in the HeLa cells were just entering the logarithmic phase.

There was no evidence that the sodium hydroxide concentration procedure was harmful to tubercle bacilli, yet it is commonly accepted that this procedure kills a large percentage of these organisms. The experimental approach that has been employed to test the toxicity of concentration procedures has generally been to prepare a suspension of tubercle bacilli from bacteriologic media and to perform colony counts before and after the concentration procedure (16–18). When it was attempted to repeat this experiment, however, microscopic examination revealed that 2 per cent sodium hydroxide caused pronounced clumping of the suspension (prepared in BSS from growth in Tween-albumin medium), and that, although a hundred fold reduction in colony count occurred quickly, it did not indicate a hundred fold reduction in viable organisms. Fenner's studies have emphasized that colony counts of tubercle bacilli are not sufficient to enumerate the viable organisms unless they are accompanied by controls of the size of bacterial groups (19).

The concentration procedures chosen for tuberculous sputum which is to be inoculated onto bacteriologic media must carry out two functions: first, the mucus must be dissolved so that the bacilli may be concentrated by centrifugation; and, second, there must be a selective killing of nonacid-fast bacteria so that the slowly developing colonies of tubercle bacilli will not be overgrown. Because the tissue cultures, which contained penicillin, did not usually allow the nonacid-fast organisms to grow, it was possible to use a mild concentration procedure which dissolved the mucus but did not appear to damage the bacteria. This allowed a study that was not possible with the bacteriologic media, namely,

the investigation of tubercle bacilli in sputum which had not been decontaminated. Such tubercle bacilli were found to exhibit approximately the same "viability rates" and speeds of growth that were shown by bacilli from other portions of the same specimens that had been treated with 2 per cent sodium hydroxide for twenty minutes.

The sensitivity of HeLa cell cultures in detecting tubercle bacilli in sputum was compared with that of the blood agar of Tarshis and associates (7). Studies comparing the sensitivities of different types of bacteriologic media can be carried out by several different approaches. The method chosen for the pesent study was to compare the number of bacilli culturable in HeLa cells with those culturable on bacteriologic medium. Tubercle bacilli were not frequently seen to have grown in HeLa cells unless the inoculum contained about 10^3 bacilli capable of growing on bacteriologic medium. It was thought that this relative insensitivity of the HeLa cell method as it was employed here could be attributed to two factors of approximately equal importance: One was the inability of the HeLa cells to extract many of the bacilli from the gross particulate matter in sputum concentrates; the other was the amount of time it took to search the cells on the coverslip for acid-fast bacilli. Examination under lesser magnifications was not much more productive except when the tissue cultures were allowed to go on to nine days in order to ensure larger groups of tubercle bacilli.

This study was confined to 61 specimens of sputum. An investigation involving larger numbers was not deemed worth while at present, due to the possibility of improvements in a technique as new as the tissue culture method here described. The disadvantages of the HeLa cell method as here described for routine diagnosis are obviously its insensitivity compared to bacteriologic media and its greater demand in time and skills. The advantages are also obvious. They are the greater speed with which the result could be obtained and the specificity of the growth patterns observed. The pronounced intracellular cording and parallel alignment differentiate pathogenic tubercle bacilli from strains of reduced virulence such as H37Ra and BCG (2), and from photochromogenic and nonphotochromogenic strains of "atypical acid-fast" bacteria (9). Strains of the saphrophytes *M. phlei* and *M. smegmatis* have not grown at all in HeLa cells (3).

Summary

Tubercle bacilli in sputum concentrates when inoculated into HeLa cells showed essentially the same picture of intracellular growth as that seen with inocula prepared from bacteriologic medium.

Of the tubercle bacilli present in the concentrates, 50 to 70 per cent were judged capable of growth in HeLa cells. Generation times were estimated to be about two days between the first and third days, and one day between the third and fifth days.

No evidence was obtained that 2 per cent sodium hydroxide was harmful to the bacilli during the concentration procedure. Concentration by trypsin digestion at neutral reaction gave tubercle bacilli with substantially the same "viability rates" and generation times.

When the tubercle bacilli from sputum were inoculated into cells which had received 1,000 r of X-irradiation, they grew at about the same rates as they did in nonirradiated cells.

HeLa cell cultures were compared with bacteriologic media for their usefulness in the laboratory diagnosis of tuberculosis. The tissue cultures were less sensitive to small numbers of tubercle bacilli, and they demanded more technical skills. The result was obtained quickly, however, and the mycobacterial growth pattern was that seen only with pathogenic mammalian tubercle bacilli.

Sumario

Estudio de la Proliferación en las Células HeLa de los Bacilos Tuberculosos Procedentes del Esputo Humano

Al ser inoculados en células HeLa, los bacilos tuberculosos del esputo concentrado produjeron en el fondo el mismo cuadro de proliferación intracelular que se observa con los inóculos preparados del medio bacteriológico.

De los bacilos tuberculosos presentes en las preparaciones concentradas, a 50 a 70 por ciento se les consideró capaces de proliferación en las células HeLa. Se calculó que los tiempos de generación eran de unos dos días entre el primer y el tercer días y de un día entre el tercer y el quinto días.

No se obtuvo prueba alguna de que el hidróxido de sodio al 2 por ciento fuera nocivo para los bacilos durante el procedimiento de concentración. La concentración mediante la digestión con tripsina a reacción neutra produjo bacilos tuberculosos de "tasas de viabilidad" y tiempos de generación substancialmente idénticos.

Al inocularse bacilos tuberculosos procedentes del esputo en células que habían recibido 1,000 r de irradiación X, aquéllos proliferaron más o menos del mismo modo que en las células no irradiadas.

Los cultivos en células HeLa se compararon con los medios bacteriológicos en cuanto a utilidad para el diagnóstico de la tuberculosis en el laboratorio. Los cultivos en tejidos fueron menos sensibles a pequeñas cantidades de bacilos tuberculosos y exigieron mayor destreza técnica. Sin embargo, se obtuvo el resultado rápidamente y el patrón de la proliferación micobacteriana fué el observado únicamente con bacilos tuberculosos patógenos de mamífero.

Resume

Etude de la croissance dans les cellules HeLa de bacilles tuberculeux humains provenant de crachats

Les bacilles tuberculeux humains, obtenus à partir de crachats concentrés, inoculés dans les cellules HeLa présentaient une croissance intracellulaire d'un aspect essentiellement semblable à celui des inocula préparés à partir de milieux bactériologiques.

Dans les extraits concentrés, 50 à 70% des bacilles tuberculeux présents furent jugés capables de croître dans les cellules HeLa. L'auteur considère que le temps de reproduction est d'environ deux jours entre le premier et le troisième jour et de un jour entre le troisième et le cinquième jour.

On n'a pas constaté que l'hydroxyde de sodium ait exercé un effet nocif sur les bacilles au cours de la concentration. Le procédé de concentration par digestion par la trypsine à un pH neutre donnait des bacilles tuberculeux possédant pratiquement les mêmes "taux de viabilité" et temps de reproduction.

Quand les bacilles tuberculeux extraits de crachats étaient inoculés dans les cellules irradiées ayant reçu 1000 r, les taux de développement étaient sensiblement les mêmes que dans les cellules non soumises à l'action des rayons X.

Une étude comparative des cultures en cellules HeLa et en milieux bactériologiques a

été effectuée pour déterminer leur utilité dans le diagnostic de la tuberculose au laboratoire. Les cultures dans les tissus se sont montrées moins sensibles aux bacilles en nombre réduit et réclamaient une technique plus compliquée. Toutefois, les résultats étaient obtenus rapidement et le type de la croissance mycobactérienne était celui qui n'est observé qu'avec les bacilles tuberculeux pathogènes de mammifères.

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TISSUE CULTURE STUDIES ON RESISTANCE IN TUBERCULOSIS[1,2]

I. Normal Guinea Pig Monocytes with Tubercle Bacilli of Different Virulence

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INTRODUCTION

Resistance to tuberculosis has been demonstrated repeatedly to exist natively in certain animal species and also to be acquired by members of a susceptible race following a number of immunizing procedures (1). The mechanism of this resistance has remained a perplexing mystery. A wide variety of circulating antibodies has been recognized in the serum of immunized persons. There is every precedent that these or other antibodies should be important in the acquired resistance to this disease. As yet, however, no acceptable evidence has been presented that these antibodies play a role in the protective mechanisms which appear to function in such animals. Such evidence may be difficult to obtain. Failure to transfer resistance passively using serum from immunized animals has been repeatedly observed, even with the most persistent of efforts (2). Yet, such experiments are accomplished with difficulty in a disease as chronic as tuberculosis, and negative results cannot be accepted as conclusive.

On the other hand, some workers have claimed that monocytes (histiocytes, macrophages, mononuclear phagocytes, et cetera) from immunized animals possess a definite increase of ability to limit the multiplication of, or even to destroy tubercle bacilli (3, 4). Other workers have not been able to demonstrate these changes (5). The techniques employed in the various experiments have been different, although tissue culture techniques have predominated.

Because of the basic importance of establishing the true facts concerning the alleged cellular factors in resistance to tuberculosis as well as studying further the role of circulating antibodies, the present writers have devised certain modified techniques of monocyte tissue culture. In the first group of experiments, observations were made upon normal guinea pig macrophages containing tubercle bacilli of various strains. These cells were maintained in a culture medium of normal guinea pig serum and balanced salt solution. Future reports will deal with the behavior of monocytes from hypersensitized guinea pigs, and then from immunized guinea pigs with and without the use of "immune" serum as the culture medium.

MATERIALS AND METHODS

The macrophages were obtained from the peritoneal cavities of guinea pigs. The animals were given 1 ml. of mineral oil into the peritoneal cavity.[3] The peritoneal cavity was lav-

[1] From the Glockner-Penrose Hospital, Colorado Springs, Colorado.

[2] This investigation was supported by a research grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association, and research grant E 808 from the Institute of Allergy and Infectious Diseases of the National Institutes of Health, U.S. Public Health Service.

[3] The mineral oil which was used in this study was Standard Oil Company's Stanolind

aged eight to fourteen days later with 40 ml. of Hank's solution through a blunt, 20 gauge needle with a 20 ml. syringe. The animals were not injured by the lavage and could be used for other purposes thereafter. Twenty milliliters of the aspirated saline suspension of cells were centrifuged for five minutes at 500 to 700 r.p.m. in two 15 ml. centrifuge tubes. The supernatant was discarded and the loosely packed cells were resuspended in a total mixture of 2 ml. of 40 per cent normal guinea pig serum and 60 per cent Hank's solution containing from 50 to 100 units of penicillin per ml. The macrophages so obtained often contained very small droplets of oil in their cytoplasm. Their behavior in tissue culture appeared completely normal. Small numbers of polymorphonuclear leukocytes and lymphocytes were also obtained. All of the cell counts which were made during the course of the experiments to determine percentages of infection, incidence of cell death, et cetera, were made upon macrophages alone. If the peritoneum was lavaged earlier than eight days, excessive numbers of polymorphonuclear leukocytes were obtained; if lavaged after fourteen days, lymphocytes predominated.

Tubercle bacilli were usually obtained from the National Jewish Hospital for Tuberculosis in Denver[4]. Four to five milliliters of a homogenized suspension of bacilli, either H37Rv (strain G) or BCG (Phipps), were received via special delivery in a Tween®-albumin medium. These arrived within twelve to eighteen hours of mailing. The bacilli were essentially in a single cell suspension with rare clumps of 1 to 5 bacilli. Usually one-half of the suspension was killed by autoclaving (121°C. for twenty-five minutes). Both the live and the killed bacilli were then centrifuged at 2,400 r.p.m. for ten minutes, resuspended in Hank's solution, and recentrifuged for the same time at the same speed. The washed bacilli were then resuspended in the mixture of 40 per cent normal guinea pig serum and 60 per cent Hank's solution containing from 50 to 100 units of penicillin per ml. The above handling resulted in some additional clumping of the bacilli. Measured amounts of the resuspended bacilli were added to aliquots of macrophages and incubated at 37°C. in roller tubes, in the roller tube apparatus, for thirty to ninety minutes. Smears were usually made at twenty-minute intervals to determine the degree of phagocytosis. If extracellular bacilli were present in significant numbers at the time that the desired amount of phagocytosis was obtained, centrifugation of the cell-bacilli mixtures at 500 to 700 r.p.m. and resuspension of the macrophages in serum-Hank's medium eliminated most of the extracellular bacilli. This procedure, however, was rarely found necessary. Control macrophages without bacilli were always handled in an identical manner.

Embryo extract was prepared from beef embryos less than 20 cm. long. The muscle, heart, skin, kidney, lungs, and brain were emulsified with a blender in Hank's solution, incubated at 37°C. in a water bath for one hour, and then centrifuged at 200 r.p.m. The supernatant was divided in aliquots of 1 ml. each and stored frozen. The same technique was later used to prepare guinea pig embryo extract from embryos 5 cm. or less in length.

Normal guinea pig plasma was made with a 1:40,000 dilution of heparin as an anticoagulant. The plasma was stored frozen in 1 ml. lots.

All glassware was washed especially for tissue culture work, using the following techniques: Coverslips were scrubbed individually by hand with Bon Ami®, rinsed in running tap water, soaked in 25 per cent H_2SO_4 for one hour, and washed in running tap water for thirty to sixty minutes. Each coverslip was handled separately in five changes of tap water and five changes of distilled water. Each coverslip was also dipped separately in 95 per cent ethyl alcohol and wiped dry with a clean linen cloth. Pipettes were soaked in 25 per cent H_2SO_4 for twenty-four hours, rinsed in running tap water for thirty minutes, rinsed ten times by inversion in tap water and five times in distilled water, and then dried in the oven. All other glassware was scrubbed with soap and water, soaked overnight in

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Liquid Paraffin Heavy. Because of the heavy acid treatment used in its manufacture, surface active agents are not present in this oil. Tocopherol is present in this mineral oil in amounts of less than 10 parts per million.

[4] Supplied through the courtesy of Drs. Gardner Middlebrook and M. L. Cohn.

Aura[5] solution (one tablespoonful per gallon), and then boiled in fresh Aura solution for thirty minutes. (After this procedure had been successfully employed for nearly two years, it was discovered that, if the same glassware is repeatedly boiled in Aura solution, cells will no longer live on the glass surface, although they may do so in the plasma clots on such a surface. Glassware is no longer boiled in the Aura solution.) The glassware was then rinsed with hot running tap water, soaked in Duponal[6] solution (one-half teaspoonful per gallon) for thirty minutes, rinsed in ten to twelve complete changes of tap water, inside and out, and then rinsed in five changes of distilled water before drying in the oven. Water was distilled in a glass still. After being wrapped in muslin packs or plugged with cotton, all of the glassware was dry heat sterilized at 160°C. for one hour. All of the techniques requiring sterile precautions were carried out under an improvised hood. Almost no secondary infections of the cultures occurred.

The tissue cultures were prepared by adding one drop of normal guinea pig plasma, one drop of embryo extract, and one drop of the macrophage suspension, onto the glass surface with bent tip capillary pipettes and mixing well. This mixture clotted rapidly to form a thin film in which the cells were trapped. Cultures of each group were then made on the inside walls of special thin, Pyrex glass roller tubes[7] to permit frequent observations of the cell behavior. Ten to twenty coverslip cultures of each group of cells in each experiment were also prepared. The coverslips were placed upon a sterile glass strip and the cells were planted upon the upper surface. The coverslips had been cut to measure 25 by 12.5 mm. and fitted loosely into a 16 by 150 mm. test tube. After clotting occurred, each coverslip was inserted into a standard roller tube. About 1 ml. of a medium consisting of 40 per cent of normal guinea pig serum and 60 per cent of Hank's solution and containing 50 to 100 units of penicillin and 5 γ of streptomycin per ml. was added to each roller tube. The tubes were corked with white rubber stoppers,[8] and were incubated at 37°C. in a standard roller tube apparatus turning at the rate of four times per hour. The medium was discarded and fresh medium was added every three to five days. The presence of phenol red in the medium allowed estimations of pH changes to be made. Daily observations of the side-wall cultures were recorded. Coverslips from each group of the experiment were fixed overnight in 10 per cent formalin when indicated, usually daily. The slips with attached cultures were stained for acid-fast bacilli, using either Kinyoun's stain or a propyleneglycol carbol fuchsin method. Methylene blue was the counterstain. After staining, the coverslip cultures were dehydrated in alcohol and permanently mounted in synthetic resin on standard glass slides. Most of the experiments were studied in culture for fourteen to eighteen days, at which time overgrowth by mesothelial or connective tissue cells, or cells that resembled these, had occurred. In many cases the fibrin clot disintegrated during the fifth to tenth days and within ten to twelve days all of the cells were lost. Frequently the monocytes adhered to the glass where the clot was lost, and continued observations were possible after loss of the fibrin clots.

Monocytes living under the conditions of this technique formed beautiful pseudopods and maintained excellent morphologic appearances. Mitotic division occurred in the monocytes but was rare, while the initially rare mesothelial cell usually multiplied rapidly to form small expanding colonies after five to seven days.

In all of the experiments, each stained coverslip was studied carefully and observations on the general morphologic appearance of the monocytes, including nuclear and cytoplasmic details, size of cells, the presence of multinucleate forms, and evidence of cell migration, were recorded. Notes were made on relative numbers of living and dead cells as well as on numbers of extracellular bacilli.

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[5] Product of Calgon Company of Pittsburgh, Pennsylvania.
[6] Product of Du Pont Company, Wilmington, Delaware.
[7] Product of Corning Glass Company, Corning, New York.
[8] Product of The West Rubber Company, Phoenixville, Pennsylvania.

An arbitrary system of counting the proportion of infected cells and the number of bacilli in the cells was devised. All counts were done by the same observer. After a survey of the entire culture with the low-power objective, 100 healthy macrophages were studied with the high-power objective and the number of intracellular tubercle bacilli in each was recorded. The cells were not selected, but samples were picked at random over the entire slip. At times, certain cells had to be disregarded because of lack of clarity due to clot formation, overlaying of one cell on another, or too intense stain. Simultaneously, an additional record of the number of killed cells or mechanically burst cells was kept. The bacillary counts were recorded as the number of cells containing no bacilli, 1 to 5, 6 to 10, 11 to 20, or more than 20 bacilli per cell. These counts were not construed as being absolutely accurate but were clearly indicative of trends. Repeat counts on the same slide could vary as much as 10 to 20 per cent although usually repeat counts approximated the first.

In the first few experiments R1Ra tubercle bacilli, obtained from the Trudeau Laboratory[9] and homogenized for phagocytosis in the writers' laboratory with a Teflon® homogenizer, were added to suspensions of macrophages from normal guinea pigs. In these experiments, streptomycin was not employed in the culture medium.

In the second series of experiments, suspensions of BCG were added to macrophages from normal guinea pigs. In other experiments, the BCG was used as a control for simultaneous cultures of H37Rv bacilli in normal guinea pig macrophages. In some experiments, streptomycin was not added to the culture medium for the first four to five days.

In the third series of experiments, H37Rv (strain G) was studied in cultures of normal guinea pig macrophages. In all of these experiments cultures with suspensions of killed H37Rv bacilli were used as controls and in some instances living BCG was also employed for comparisons. In several of these experiments also, streptomycin was not added to the culture medium for the first four to five days.

Results

The few experiments done with the R1Ra strain of avirulent tubercle bacilli in macrophages from normal guinea pigs clearly showed that the rate of the R1Ra bacterial multiplication was much slower than that of the H37Rv strain. Final conclusions concerning the possible complete inhibition of intracellular bacillary multiplication by the macrophage could not be reached since streptomycin was not added to the medium in these experiments. Continued phagocytosis of viable and multiplying extracellular bacilli was therefore probable. It was clear, however, that the macrophages survived and behaved as well with large numbers of intracellular R1Ra bacilli in a medium also containing large numbers of living bacilli as did the control cells. Migration, as demonstrated by pseudopod formation and other evidences of cell health such as cytoplasmic granularity or nuclear detail, remained as satisfactory in the cultures with bacilli as in those without bacilli (figure 1). Only very rarely did a macrophage become so distended with bacteria that the cell became round and the nucleus obscured. Burst cells were not observed.

Experiments with BCG: In three experiments with BCG (Phipps), the initial cell infection was quite different in extent, doubtlessly accounting for the varied effects on the cells. In one experiment, streptomycin was not added to the medium until the fifth day. The total cell infection at the start of the experiment

[9] Courtesy of Dr. William Steenken.

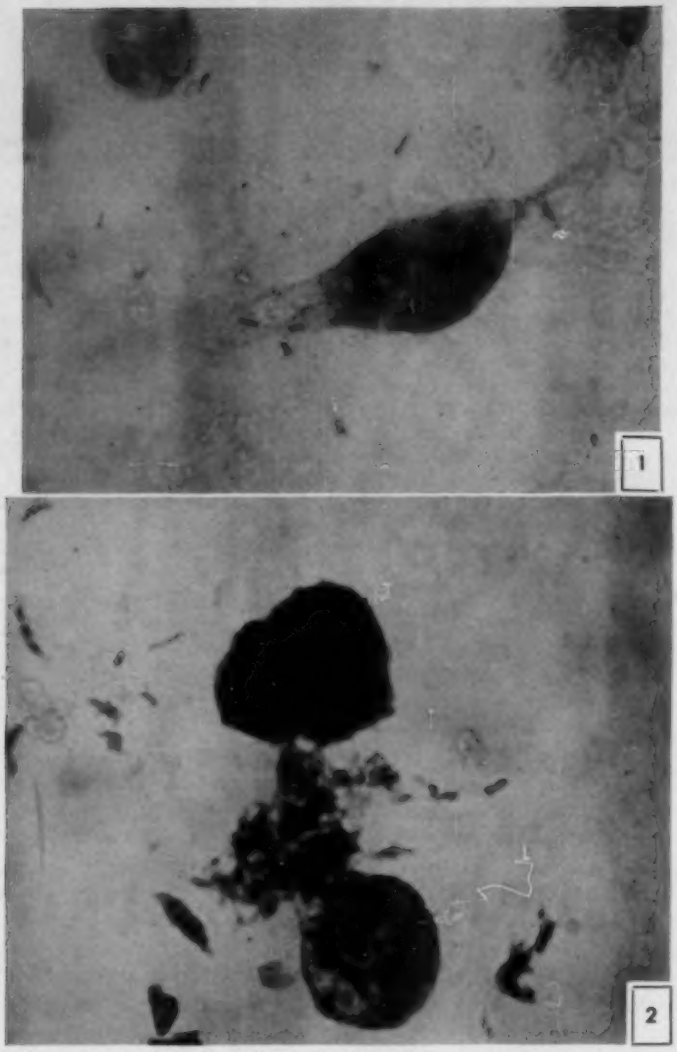

FIG. 1. Monocyte loaded with tubercle bacilli of R1Ra strain showing normal pseudopods on the tenth day.

FIG. 2. Monocytes loaded with tubercle bacilli of BCG strain showing two rounded inactive cells and one burst cell on the tenth day.

TABLE 1

TISSUE CULTURE OF NORMAL GUINEA PIG MONOCYTES WITH BGG*

Day	Bacilli per Cell in Per Cent			Total Per Cent of Cells Infected	Killed Cells	Appearance of Infected Living Cells
	1-5	6-20	>20			
1	53	33	5	91	25 to 30 per cent	Excellent
3	22	49	23	94	0	Excellent
6†	5	31	64	100	5 to 10% "bursting"	Excellent
10	10	38	52	100	Many dead, many "bursting"	Excellent

* No streptomycin in medium for five days.
† Streptomycin 5 γ per ml. added to medium.

TABLE 2

TISSUE CULTURE OF NORMAL MONOCYTES WITH BCG

Day	Bacilli per Cell in Per Cent			Total Per Cent of Cells Infected	Killed Cells	Appearance of Infected Living Cells
	1-5	6-20	>20			
1	64	8	—	72	1 to 2%	Good
3	54	21	—	75	0	Excellent
6	62	7	—	69	0	Excellent
10	58	9	—	67	0	Excellent
15	52	28	—	80	0	Fair*

* Same as control cultures

TABLE 3

TISSUE CULTURE OF NORMAL MONOCYTES WITH BCG

Day	Bacilli per Cell in Per Cent			Total Per Cent of Cells Infected	Killed Cells	Appearance of Infected Living Cells
	1-5	6-20	>20			
0	65	17	—	82	0	Excellent
1	60	14	—	74	0	Excellent
3	65	26	—	91	0	Excellent
6	59	25	—	84	0	Excellent
10	60	23	—	83	0	Good

was high (table 1) and the number of cells with more than five bacilli per cell was also relatively high. In this experiment it was apparent that macrophages initially containing more than five BCG bacilli might die relatively rapidly. These cells apparently died of some directly injurious effect from the intracellular bacteria and not of mechanical bursting which only occurred later. The initial effect or "shock" killed perhaps 25 to 30 per cent of the cell population. Control cultures showed no such initial cell death, since after a lag of six to twelve hours normal macrophage cultures were composed almost entirely of active cells. Most of the cell death in the cultures with bacilli occurred within twenty-four hours; thereafter, for as long as four to five days, the remaining monocytes

improved and seemed to behave normally even with large numbers of intracellular bacilli. Almost no extracellular bacilli were found during this time. Then, excessive numbers of cells died in comparison with the controls. Many of the dying cells seemed to literally burst from the increase of intracellular bacilli, as though from mechanical force (figure 2). Yet the cells exhibited entirely normal cell behavior and morphology until overwhelming numbers of intracellular bacilli had accumulated. In this regard, the cell death which occurred late in the experiment was quite different from that which took place during the first 48 hours of the experiment.

In two other experiments, in which 5 γ of streptomycin per ml. were added to the culture medium at the beginning, the initial infection was less severe although the total number of cells infected was high (tables 2 and 3). In both of these

TABLE 4

TISSUE CULTURE OF NORMAL MONOCYTES WITH H37Rv

Day	Bacilli per Cell in Per Cent			Total Per Cent of Cells Infected	Killed Cells	Appearance of Infected Living Cells
	1–5	6–20	>20			
0	63	8	—	71		
1	63	12	—	75	10 to 20%	Fair*
3	44	55	1	100	0	Excellent
6	67	25	2	94	rare	Excellent
9	69	14	8	91	5 to 10 per cent "bursting"	Good
15	32	59	5	96	5 to 10 per cent "bursting"	Fair†

* Not as active or healthy as controls

† Probably as good as controls

TABLE 5

TISSUE CULTURE OF NORMAL GUINEA PIG MONOCYTES WITH KILLED H37Rv

Day	Bacilli per Cell in Per Cent			Total Per Cent of Cells Infected	Killed Cells	Appearance of Infected Living Cells
	1–5	6–20	>20			
0	49	31	—	80	0	Good*
1	45	20	—	65	0	Excellent
3	55	11	—	66	0	Excellent
5	48	28	—	76	0	Excellent
7	40	3	—	43	0	Excellent
9	46	6	—	52	0	Excellent
11	51	15	—	66	0	Excellent
13	49	24	—	73	0	Excellent

* Same as controls

TABLE 6

TISSUE CULTURE OF NORMAL GUINEA PIG MONOCYTES WITH H37Rv

Day	Bacilli per Cell in Per Cent			Total Per Cent of Cells Infected	Killed Cells	Appearance of Infected Living Cells
	1-5	6-20	>20			
0	14	65	19	98		
1	42	47	1	90	40 per cent	Fair*
3	19	62	18	97	60 per cent "bursting"	Excellent
5	11	52	37	100	80 per cent "bursting"	Good*
8	24	54	11	87	Progressive increase in dead cells and "bursting"	Good*
12	5	46	49	100	Progressive increase in dead cells and "bursting"	Fair*

* Not as active or healthy as controls

experiments the initial cell injury was trivial, intracellular multiplication of the bacilli was not apparent, and cell death consequent to the intracellular bacilli did not seem to occur.

Experiments with H37Rv: Several experiments demonstrated the necessity of having streptomycin in the culture medium from the start of incubation when the H37Rv strain of bacilli was used. In earlier experiments without streptomycin, the rapid extracellular multiplication of bacilli permitted continued phagocytosis of large numbers of bacilli and often resulted in complete cell death within three to five days. With the use of 5 γ of streptomycin per ml., however, the effect of the bacteria upon the cells seemed directly related to the severity of the initial cell infection. In one experiment with a rather high total infection but with few bacilli per cell (table 4), the cell death was reduced to approximately that seen in much heavier infections with BCG. Excellent macrophage activity was continually observed despite the gradual increase to rather large numbers of intracellular bacilli with cord formations noted in many monocytes. Evidence of intracellular multiplication with the H37Rv bacilli was always more prominent than with the BCG bacilli. No intracellular multiplication or cell injury occurred in control experiments with killed H37Rv bacilli (table 5). When heavier infections were produced with live H37Rv bacilli, macrophage death occurred rapidly (table 6).

In all of the experiments, the most profound injury to the cells seemed to occur during the first twenty-four hours (figure 3). The cells which survived were those with fewer intracellular bacilli or with no bacilli. The surviving cells rapidly improved in activity and assumed normal morphology. By the third day, cultures of macrophages with intracellular H37Rv bacilli totaling 80 to 90 per cent infection, although of low numbers per cell, might appear as excellent as those cells without bacilli or as those with intracellular killed organisms (figure) 4.

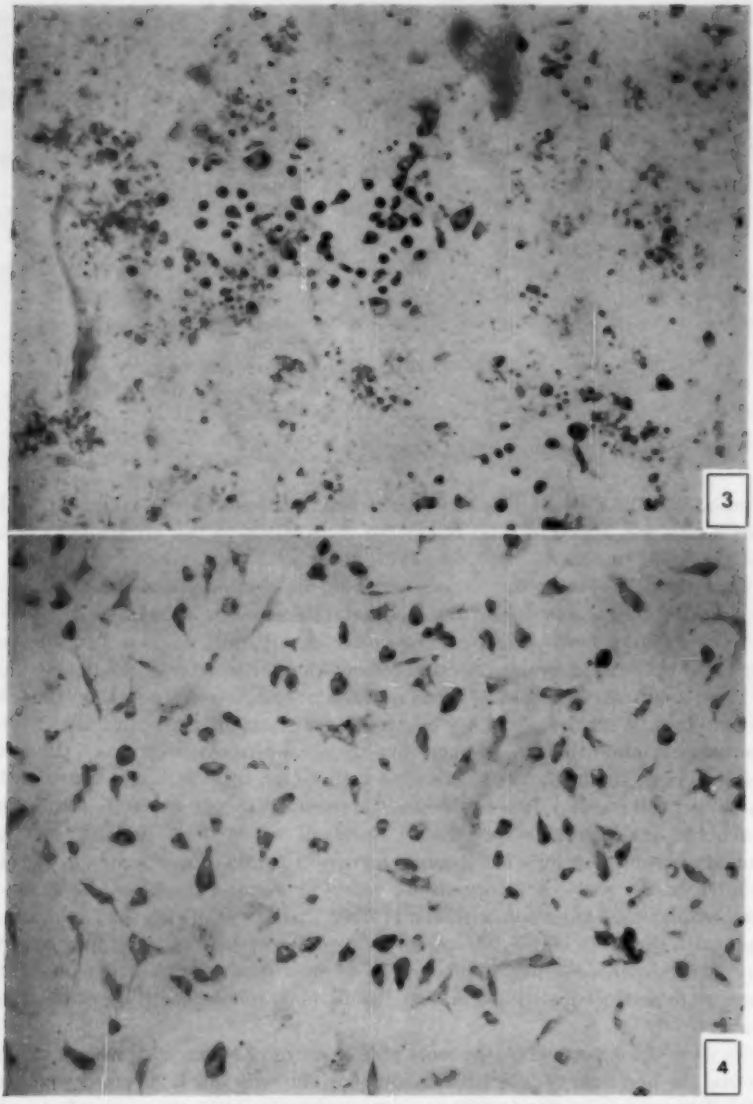

FIG. 3. An exceedingly heavy infection of monocytes by H37Rv on the first day with many dead cells and beginning activity of cells with fewer intracellular bacilli.

FIG. 4. Light infection of monocytes by H37Rv on the third day with normal activity of cells.

Discussion

These initial experiments have confirmed certain basic observations already made by Suter (6) and Mackaness (7). In the present studies, also, it has been seen that, while monocytes from normal animals of a species susceptible to tuberculosis are quite unable to inhibit the intracellular multiplication of a virulent strain of tubercle bacillus and are killed in relatively large numbers by such bacilli, these cells are able to survive and behave normally with large numbers of intra- and extracellular avirulent organisms. Attenuated bacilli, such as BCG, lie somewhere between, as far as the cell-parasite relationship is concerned. The present experiments also indicate that there are important quantitative relationships between the host cells and tubercle bacilli. When, for example, a very heavy infection with BCG was provided, the monocytes succumbed initially at a rapid rate. The monocytes in such experiments also died at an excessive rate as the experiment progressed, apparently as an effect of the increase in numbers of intracellular bacilli. In this case, then, monocytes reacted to large numbers of attenuated organisms in much the same fashion as to smaller numbers of the virulent tubercle bacilli in other experiments. These observations are what would be predicted.

The assumption that the normal guinea pig monocyte was able to inhibit the intracellular multiplication of attenuated bacilli in those experiments in which the initial level of infection was low depends upon the acceptance of certain observations made by Mackaness (8). In his experiments, streptomycin, in slightly larger concentrations than those used in the present experiments, did not penetrate the cell. In the same experiments in which BCG bacilli were unable to multiply, the H37Rv strain of bacilli was found to multiply intracellularly despite the same concentration of streptomycin in the medium and essentially the same initial level of infection. Nevertheless, intracellular BCG may be more susceptible to small quantities of streptomycin which may penetrate the cell at this low concentration in the medium. Since penicillin was present in all of the experiments, the presence of this drug in the rather high concentration used could not explain the differences.

The present findings are also in agreement with those of Mackaness and associates (7) who described the rate of intracellular multiplication of virulent bacilli as occurring much faster than that of attenuated organisms when the initial level of infection was similar.

In several other aspects the present observations differ from those recorded by other investigators. The ability of certain monocytes to migrate in tissue cultures and to maintain a completely normal morphologic appearance for eight to fourteen days with large numbers of virulent tubercle bacilli in their cytoplasm is of interest. That the bacilli were living and probably multiplying is indicated by the development of cords and by the rapid increase in numbers of intracellular bacilli. Whether the bacterial virulence had been altered in any way is not known.

Allgower and Bloch (9) observed no migration of polymorphonuclear leukocytes containing virulent bacilli. These workers, however, studied the leukocytes

for only twelve to fourteen hours. Actually, in the present experiments during the first eighteen to twenty-four hours the monocytes which contained virulent bacilli also showed poor migratory activity in comparison with the controls. There seemed to be a period of initial "shock" which was not present in control cultures of cells without bacilli or in those containing dead bacilli. During these first few hours cell death was apt to be most rapid. It was clearly seen that the cells with the most intracellular bacilli were the ones that died. Monocytes with no intracellular bacilli or those with less than five bacilli usually survived, either in the quiescent rounded condition or with prompt production of pseudopods. Typically, by the third or fourth day all of the cells that survived the initial shock were active and exhibited excellent morphologic appearances regardless of the numbers of intracellular bacilli which had accumulated by this time.

This initial period of shock, with excellent recovery of living cells, was not mentioned by Suter or Mackaness but has occurred so regularly in the present experiments that the writers believe it to be a basic phenomenon of the cell response to the ingested bacilli. During the initial preparation of the cultures, the monocytes were doubtlessly subjected to certain trauma, i.e., aspiration from the peritoneal cavity, centrifugation, et cetera, and therefore, the cells might be more susceptible to the effects of intracellular infection during the first few hours. Nevertheless, it seems that many of the cells made a satisfactory adjustment to the presence of the intracellular bacilli, allowing the resumption of normal activity and morphologic appearance after the initial effects of the shock had caused considerable inhibition of activity. The surviving cells were not located in any particularly advantageous area of the culture but were found throughout the clot, intermingled with those cells which were dying. Studies to investigate possible changes in the medium such as oxygen and carbon dioxide content, which might have occurred at this time to inhibit the bacilli and to aid the cell, have not yet been performed. The occasionally observed changes in pH did not appear significant in this respect.

Some of the infected cells which survived now doubtlessly contained bacilli which had been released from cells killed during the first few hours or days, or bacilli which had been still extracellular at the start of the experiment. In each case, these bacilli would be organisms which had been exposed to streptomycin and therefore, perhaps, were no longer pathogenic. But cord formation in many cells probably indicated persistently virulent organisms multiplying intracellularly without immediate injury to the cell. Of greater importance was the fact that the remaining cell population, after the initial cell death, was always much greater than could be explained by the survival only of initially uninfected macrophages.

With the techniques employed, when less than 10 per cent of the cells were initially infected, it was difficult to see evidence of bacillary multiplication. Under such circumstances it was observed that some infected cells died during the first forty-eight hours, but thereafter most of the intracellular bacilli which were found usually did not appear to be multiplying and were assumed to be organisms re-

leased from killed cells and rephagocytized. To be sure, some cells with multiplying bacilli were found, but in such small numbers as to be considered insignificant. It was believed necessary, therefore, to commence the experiment with a relatively high percentage of infected cells—30 to 60 per cent—so that appreciable numbers of the cells containing bacilli phagocytized prior to the addition of streptomycin remained for study after the initial cell death. It was obviously desirable to have as few bacilli per cell as possible in order to reduce to a minimum the initial cell death.

After recovery from the initial effects of the intracellular bacilli, the cells which survived continued to manifest essentially normal behavior until intracellular increases of bacilli seemed literally to burst the cell as by mechanical pressure rather than from the "toxic" effect that had killed the cells during the first 48 hours.

These observations are in sharp contrast to those made by other investigators. While Mackaness (8) has noted the survival of rabbit macrophages with many intracellular bacilli of the H37Rv strain, this occurred only in streptomycin concentrations of 12.5 mg. per ml., concentrations which he showed had resulted in intracellular inhibition of the bacilli. In his experiments with streptomycin concentrations of 6.25 mg. per ml., the monocytes were completely lysed in forty-eight hours. Suter (6) stated that normal guinea pig cells were killed within five days by intracellular bacilli of the H37Rv strain. In Suter's experiments only 8 per cent of the cells were initially infected. Rephagocytosis occurs rapidly in a culture reasonably well populated with active cells, and the complete disappearance of killed cells also takes place quickly. As mentioned in the present study, the excellence of the cultures on the third day usually contradicted the severe toxic picture seen on the first day. These results are rather in agreement with the concept that the initial spread of bacilli in the lungs of patients with primary tuberculosis may occur in living and active monocytes that migrate to local lymph channels or otherwise reach the hilar lymph nodes.

The use of mineral oil as the provocative substance to obtain the monocytes has been criticized. In this regard the present results may be better compared with Mackaness' results who also used this substance, although in much greater quantities. The objection that the mineral oil might contain surface active agents which could inhibit the intracellular bacilli in the manner described by Comforth and associates (10) is not justified. The mineral oil which has been used in the present experiments did not contain surface active agents except for weakly active tocopherol in amounts less than 10 parts per million. Occasionally multiplying bacilli seemed to be contained within a round droplet of mineral oil. Neighboring cells might show intracellular cord-forming bacilli lying immediately adjacent to small droplets of oil.

The most serious deficiency in tissue culture studies of the resistance of monocytes to tubercle bacilli is obvious. Techniques to study monocytes in tissue culture for months have not been perfected. Such methods might show other differences in the resistance of monocytes to virulent and attenuated bacilli.

For, the initial behavior of these organisms *in vivo* is remarkably similar and it is only after three or four weeks that significant differences in the behavior of various organisms in the living animal may become clearly discernible.

Summary

Tissue culture experiments have been carried out, employing normal guinea pig monocytes infected with various strains of tubercle bacilli.

As previously reported by others, virulent bacilli destroyed the monocytes more rapidly than attenuated bacilli and were able to multiply intracellularly in the surviving cells at a more rapid rate. Heavy infection levels of attenuated bacilli had the same effect on normal monocytes as light infections by virulent organisms.

The most severe effect of all bacilli on cells occurred within the first forty-eight hours. This initial cell death appeared to be the result of a "toxic" effect of relatively small numbers of intracellular bacilli. Thereafter, even with virulent organisms, some monocytes survived and migrated well with large numbers of intracellular multiplying tubercle bacilli. The final death of such cells in tissue culture seemed to be a consequence of mechanical "bursting" of the cell.

Sumario

Estudios en Cultivos de Tejidos sobre la Resistencia en la Tuberculosis: I. Monocitos Normales de Cobayos con Bacilos Tuberculosos de Distinta Virulencia

En estos experimentos con cultivos de tejidos, se emplearon monocitos normales de cobayos, infectados con varias cepas de bacilos tuberculosos.

Según ya han comunicado otros, los bacilos virulentos destruyeron los monocitos más rápidamente que los atenuados y pudieron multiplicarse con más velocidad en las células sobrevivientes. Las intensas infecciones con bacilos atenuados ejercieron el mismo efecto sobre los monocitos normales que las infecciones leves con microbios virulentos.

El efecto más intenso de todos los bacilos sobre las células tuvo lugar durante las primeras cuarenta y ocho horas. Esta mortalidad celular inicial pareció ser la consecuencia de un efecto "tóxico" de cantidades relativamente pequeñas de bacilos intracelulares. A partir de ahí, aun tratándose de gérmenes virulentos, algunos monocitos sobrevivieron y migraron sin dificultad con grandes cantidades de bacilos tuberculosos intracelulares en vías de multiplicación. La muerte final de esas células en los cultivos de tejidos pareció ser a consecuencia de la "explosión" mecánica de aquéllas.

Resume

Recherches sur la résistance à la tuberculose par la culture de tissus: I. Monocytes de Cobaye sain renfermant des bacilles tuberculeux de virulence variée

Des expériences de culture de tissus ont été effectuées en utilisant des monocytes de cobayes inoculés avec diverses souches de bacilles tuberculeux.

Ainsi que d'autres auteurs l'ont déjà signalé, les bacilles virulents détruisaient les monocytes beaucoup plus rapidement que les bacilles atténués et ils avaient la capacité de se multiplier à une vitesse beaucoup plus grande dans les cellules qui survivaient. L'infection avec des doses élevées par des bacilles atténués avait le même effet sur les monocytes normaux que l'infection à dose faible avec des germes virulents.

Tous ces bacilles exerçaient leur effet le plus intense sur les cellules dans les premières quarante-huit heures. La destruction cellulaire initiale para'ssait due à l'effet toxique d'un nombre relativement faible de bacilles intracellulaires. Ensuite, même lorsque les

germes étaient virulents, quelques monocytes survivaient et se déplaçaient facilement malgré la présence d'un grand nombre de bacilles tuberculeux intracellulaires en multiplication. Il semble que la destruction finale de ces cellules dans les tissus de culture soit la conséquence d'un éclatement de la cellule sous l'influence de la distension.

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ANTIGENIC RELATIONSHIPS BETWEEN NINETY-EIGHT STRAINS OF MYCOBACTERIA USING GEL-DIFFUSION PRECIPITATION TECHNIQUES[1, 2]

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INTRODUCTION

The advent of gel-diffusion precipitation techniques for antigen analysis in 1948 (1) opened a new avenue of approach to the problem of the determination of antigenic relationships between bacteria. This approach, however, remained unexploited for the mycobacteria until 1956 when Parlett and Youmans (2), by the use of a modified Ouchterlony double-diffusion method, reported that 42 mycobacterial strains could be divided into four antigenic groups.

In the technique that was used for this earlier study, the constituents of concentrated mycobacterial culture filtrate preparations were allowed to diffuse through neutral agar from one reagent reservoir, and rabbit antiserum was allowed to diffuse from an adjacent reagent well. In those areas in the central neutral diffusion field where suitable relative concentrations of antigen and antibody occurred, providing an adequate volume of antibody was present, visible insoluble antigen-antibody complexes formed. According to the interpretation of this phenomenon as proposed by Oudin (1, 4), Ouchterlony (3), and Becker and Munoz (5), each precipitate band is representative of one or more antigen-antibody systems.

Subsequent attempts to improve the method led to the following observations. A greater total of sharply defined precipitate bands was obtained when washed viable mycobacterial cells suspended in an agar solution were used as a source of diffusible antigen in place of the concentrated culture filtrate antigen preparation originally employed. With the cell suspension as an antigen source, a maximum of six precipitate bands was noted with certain cultures while, in a comparable system in which the filtrate preparation was used, a total of only four bands was observed. This use of viable cells as a source of diffusible antigen was based upon a method of agar double-diffusion precipitation devised by Chen and Meyer (6) for use with *Pasteurella pestis*.

In the current investigation of antigenic relationships, 98 mycobacterial strains have been investigated, using the double-diffusion precipitation method. Viable bacterial cell suspensions have been substituted for the concentrated culture filtrate preparations previously employed (2).

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[2] This investigation was supported, in part, by a research grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association.

TABLE 1

NATURE AND SOURCE OF THE 98 MYCOBACTERIAL CULTURES STUDIED BY THE AGAR DOUBLE-DIFFUSION PRECIPITATION TECHNIQUE

I. Twenty *M. tuberculosis* var. *hominis*
a) Known strains:
H37Rv
H37Ra
RlRv
JH6
JH16
b) Recently isolated strains:

H-144	L-1
H-398	L-3
H-484	L-5
H-485	L-10
H-486	L-11
H-489	L-15
H-499	L-26
	L-32

II. Fifty-four "atypical" acid-fast strains:

R-1-P	R-22-P	R-217-P	L-18-N
R-2-N	R-23-N	R-222-P	L-20-O
R-3-N	R-24-P	R-224-P	L-23-O
R-5-O	R-25-N	R-225-N	L-24-P
R-6-O	R-35G-P	R-227-P	L-25-O
R-7-N	R-80G-P	L-4-N	L-27-P
R-8-P	R-118-O	L-6-P	L-29-P
R-15-O	R-123-P	L-7-P	L-31-O
R-16-P	R-160-N	L-8-O	L-33-O
R-17-P	R-170-N	L-9-P	L-34-P
R-18-P	R-171-N	L-12-O	L-35-O
R-19-O	R-182-P	L-14-P	L-36-P
R-20-N	R-198-N	L-16-O	R-223-P
R-21-P	R-212-P	L-17-P	

III. Four *M. tuberculosis* var. *bovis*
BCG-1
BCG-4
4228-2
4228-4

IV. Two *M. avium*
Kirchberg
Sheard

V. Acid-fast saprophytes
M. smegmatis
607
G-393, *M. smegmatis*
G-573, *M. smegmatis*
G-10143, *M. smegmatis*
M. butyricum
M. ranae
M. phlei
G-5, *M. phlei*
G-8a, *M. phlei*

VI. Miscellaneous strains
M. ulcerans
M. balnei
G-927, *M. marinum*
G-389, *M. fortuitum*
G-6841, *M. fortuitum*
G-8573, *M. fortuitum*
Vole

P = photochromogen N = nonphotochromogen O = scotochromogen

METHODS AND MATERIALS

Cultures: The 98 strains of mycobacteria and the sources from which they were obtained are given in table 1. Thirty-three atypical cultures were obtained from Dr. Ernest Runyon[3] and are identified in the remainder of this report by the prefix (R-). Twenty-nine cultures were supplied through the kindness of Dr. William Lester[4] and are identified by the prefix (L-). Dr. Ruth Gordon[5] supplied 10 strains of rapidly growing acid-fast bacilli (3). These latter strains are designated by the prefix (G-). Seven freshly isolated typical *Mycobacterium tuberculosis* var. *hominis* cultures were supplied by Dr. Nelda Holmgren[6] and

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[4] Suburban Cook County Tuberculosis Sanatorium, Hinsdale, Illinois.
[5] Department of Microbiology, Rutgers University, New Brunswick, New Jersey.
[6] Veterans Administration Research Hospital, Chicago, Illinois.

TABLE 2

MYCOBACTERIAL AND FUNGAL CONCENTRATED CULTURE FILTRATE PREPARATIONS USED FOR THE PREPARATION OF ANTISERA

I. *M. tuberculosis* var. *hominis*		
H37Rv		
H37Ra		
RIRv		
II. "Atypical" acid-fast bacilli		
R-1-P	R-21-P	
R-2-N	R-22-P	
R-3-N	R-23-N	
R-5-O	R-25-N	
R-6-O	R-35G-P	
R-7-N	R-80G-P	
R-8-P	R-118-O	
R-15-O	R-212-P	
R-16-P	R-222-P	
R-17-N	R-224-P	
R-18-P	L-31-O	
R-19-O	L-15	
R-20-N	L-16	
	L-5-N	
III. *M. tuberculosis* var. *bovis*		
BCG-1		
4228-2		
IV. Acid-fast saprophytes		
607		
M. smegmatis		
M. butyricum		
M. ranae		
M. phlei		
G-5, *M. phlei*		
V. *M. avium*		
Kirchberg		
Sheard		
VI. Miscellaneous strains		
G-927, *M. marinum*		
M. ulcerans		
G-389, *M. fortuitum*		
VII. Fungi		
Nocardia pelliteriae		
Nocardia braziliensis		
Penicillium notatum		
Candida albicans		

P = photochromogen N = nonphotochromogen O = scotochromogen

are identified by the prefix (H-). The balance of the strains studied are the same as those listed in the earlier reports (2).

Antisera: Rabbit antisera prepared in the same way as in the first study (2) were employed. A list of the 43 mycobacterial and 4 fungal concentrated culture filtrate preparations used for the preparation of antisera in rabbits is given in table 2.

Modified double-diffusion precipitation technique: The method used for preparation of agar double-diffusion precipitation plates was a modification of the Ouchterlony technique used in the preliminary investigation of antigenic relationships (2). The size of the Petri plate, the arrangement of reagents therein, and the substitution of suspensions of mycobacterial cells for culture filtrate antigen preparations previously employed (2) constituted the principal changes.

Chemically clean standard Pyrex Petri plates were prepared as follows: Twenty milliliters of 2.5 per cent melted purified agar in 0.9 per cent saline were poured into sterile plates and allowed to harden. Upon solidification of this layer, seven reagent reservoirs were cut from the plate according to the diagram given in figure 1. The center reagent well was then filled with a solution consisting of antisera diluted in a 4:1 ratio of antibody to agar. The 6 peripheral wells then were filled with appropriately diluted ground and washed cell suspensions. These suspensions were harvested, ground, and standardized according to the method given by Youmans and Youmans (7). Using modified Proskauer and Beck agar medium, the suspensions were diluted to obtain a final concentration of 2.5 mg. of cells (wet weight) per ml. of agar.

Following preparation, the plates were placed in copper Petri dish cans in a 37°C. hot

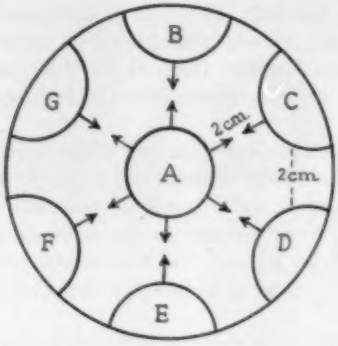

FIG. 1. A schematic representation of the modified Ouchterlony plate as it has been used for antigenic analysis of agar suspensions of viable mycobacterial cells.

air incubator and observed daily for a period of three weeks. Incubation of *Mycobacterium ulcerans* was carried out at approximately 33°C.

Cultures and antisera were labeled with code numbers, and all plates were prepared and reactions were recorded according to the code numbers. Upon termination of individual experiments, the assays were decoded and the data analyzed. This served to remove any subjectivity from the interpretation of the reactions as they appeared in the diffusion plates.

RESULTS

Repeated assays using all possible combinations of cultures and antisera were carried out. From a comparison of the reactions observed in these assays, the 98 strains could be divided into eight groups. These groups, presented in table 3, are compiled from four or more assays with each organism. In all cases the greatest number of bands observed at any one time has been recorded for each system.

Group I is comprised of 64 cultures. Nineteen of these cultures are known human strains; 42 are atypical strains, 27 of which are photochromogenic; 6 are nonphotochromogenic; and 9 are scotochromogenic cultures. In addition the avian strain, Kirchberg, and the strains *M. balnei* and vole belong to this group. All microorganisms in this group produced six separate bands of precipitate with homologous antisera and reacted equally well with all of the heterologous antisera within the group.

Group II appears to be related to *Group I* in that member strains of *Group II* cross-reacted with all of the *Group I* antisera although they produced only four bands of precipitate. Homologous reactions with the *Group II* strains also produced only four bands of precipitate. In summary, *Group II* is comprised of 5 "atypical" strains (4 scotochromogenic and one nonphotochromogenic), 4 bovine strains, and the avian strain, Sheard.

Group III is composed of saprophytic strains of mycobacteria. Strains of this group produced six bands of antigen antibody precipitate with all antisera pre-

TABLE 3

SEROLOGIC GROUPING OF 98 MYCOBACTERIAL STRAINS BASED UPON RESULTS OBTAINED FROM A MODIFIED AGAR DOUBLE-DIFFUSION PRECIPITATION METHOD

Group I (6 Precipitate Bands)		Group II (4 Precipitate Bands)	Group III (6 Precipitate Bands)	Group IV — (4 Precipitate Bands)	Group V (6 Precipitate Bands)	Group VI (2 Precipitate Bands)	Group VII (6 Precipitate Bands)	Group VIII (4 Precipitate Bands)
H37Rv	R-217-P	R-19-O	607	*M. phlei*	*M. ulcerans*	R-15-O	G-389,	G-927,
H37Ra	R-222-P	R-2-N	*M. butyricum*	G-5, *M. phlei*	R-24-P	R-17-P	*M. fortuitum*	*M. marinum*
RlRv	R-223-P	R-5-O	*M. ranae*	G-8a, *M. phlei*	L-15			
JH6	R-224-P	R-6-O	*M. smegmatis*		L-16-O		G-6841,	R-35G-P
JH16	R-225-N	R-215-O			L-31-O		*M. fortuitum*	R-20-N
H-144	R-227-P	BCG-1	G-393,					R-23-N
H-398	L-1	BCG-4	*M. smegmatis*				G-8573,	
H-484	L-3	4228-2					*M. fortuitum*	
H-485	L-4-N	4228-4	G-573,					
H-486	L-5	Sheard	*M. smegmatis*					
H-489	L-6-P							
H-499	L-7-P		G-10143,					
Kirchberg			*M. smegmatis*					
Balnei	L-8-O							
Vole	L-9-P							
R-1-P	L-10							
R-3-N	L-11							
R-7-N	L-12-P							
R-8-P	L-17-P							
R-16-P	L-18-N							
R-19-O	L-20-O							
R-18-P	L-23-O							
R-21-P	L-24-P							
R-22-P	L-25-O							
R-25-N	L-26							
R-72-P	L-27-P							
R-80-P	L-29-P							
R-118-O	L-32							
R-123-P	L-33-O							
R-160-P	L-34-P							
R-184-P	L-35-O							
R-212-P	L-36-P							
	L-14-P							

P = photochromogen N = nonphotochromogen O = scotochromogen

pared against the mycobacteria in *Group III*, but did not react with antisera prepared against the strains which appear in the remaining six groups.

Group IV consists only of the 3 *M. phlei* strains. These 3 strains produced 4 precipitate bands with anti-phlei rabbit serum only and did not react with any other antisera.

Groups V and *VI* exhibit much the same type of inter-relationship as exists between *Groups I* and *II*, since the cultures given as *Group V* produced six precipitate bands with homologous antisera but produced only two bands of precipitate with the antisera prepared against cultures listed in *Group VI*. The 2 strains which represent *Group VI* produced a maximum of only two bands in homologous antisera and only two bands of precipitate with the antisera of *Group V*.

Serologic *Group VII* is comprised of 3 strains of *Mycobacterium fortuitum*. The fortuitum cultures produced six bands of precipitate only with antisera prepared against *M. fortuitum*.

Three atypical strains of acid-fast bacilli and the *M. marinum* strain, G-927, have been put together to form *Group VIII* in table 3 mainly for the sake of convenience. Each of these cultures proved to be serologically separate and distinct from any of the others and produced four precipitate bands with homologous antiserum only. However, it may be found in the future that each of these cultures is representative of a separate serologic group.

FIG. 2. R-118 antiserum in the center reservoir as it was precipitated by antigens from the six cell suspensions in the peripheral reagent wells after eleven days of incubation at 37°C.

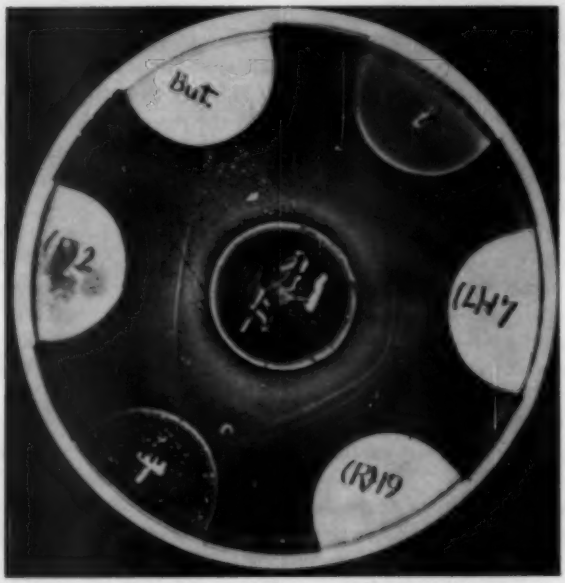

FIG. 3. R-18 antiserum as it produced the characteristic four antigen-antibody complexes with the three *Group II* strains L-17, R-19, and R-2. Failure of precipitation to develop between this antiserum and cell suspensions of *M. butyricum*, *M. phlei*, and strain 607 is evident.

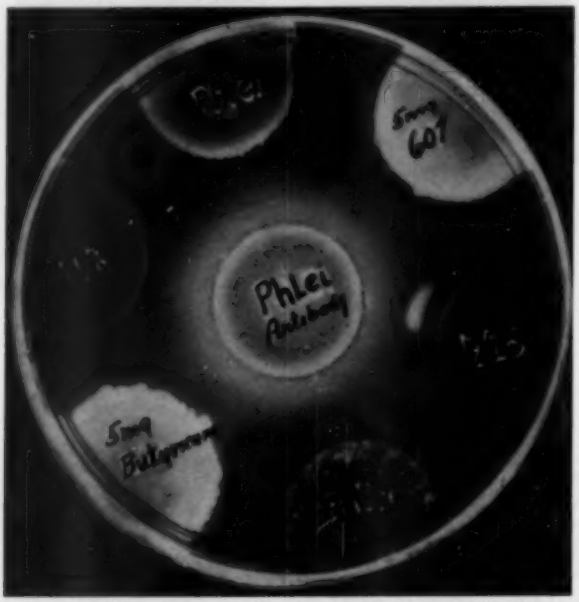

FIG. 4. A typical homologous precipitation reaction between anti-phlei serum and antigens from a cell suspension of *M. phlei* cells. No precipitation occurred between cell suspensions of cultures 607, R-225, R1Rv, *M. butyricum*, or R-118.

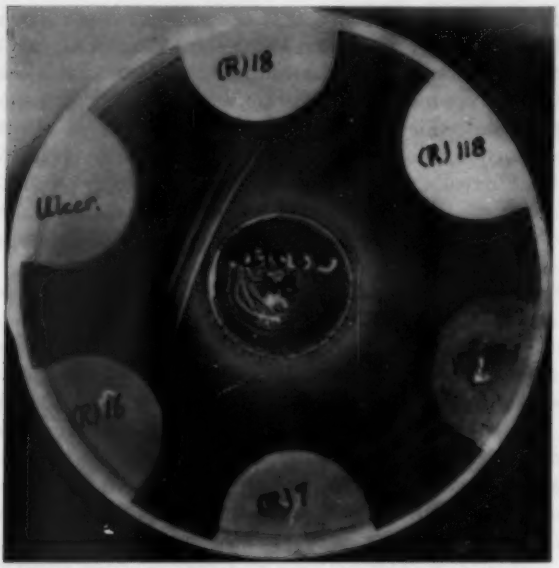

FIG. 5. A typical homologous precipitation reaction between anti-ulcerans serum and antigens from a cell suspension of *M. ulcerans*. No precipitation occurred between this antiserum and cell suspensions of cultures R-18, R-118, H37Rv, R-7, or R-16.

Illustrations of some of the reactions which suggested the serologic grouping presented herein may be seen in figures 2 through 5.

In figure 2 the *Group I* R-118 antiserum is shown in plates with cell suspensions of *Group I* strain R-21 and *Group II* strains R-5, L-17, R-19, and the bovine strain BCG and avian strain Sheard. This photograph was taken after eleven days of incubation, at which time all reactions were maximal and complete except for strains R-5 and L-17 which became complete eight days later.

In figure 3 the 4 precipitate bands are demonstrated which were obtained when *Group I* strain R-18 antiserum reacted with *Group II* strains L-17, R-19 and R-2. Failure of precipitation to occur between cell suspensions of strains 607, *M. butyricum*, and *M. phlei* and this antiserum is evident.

A typical homologous *Group V* phlei reaction is shown in figure 4. There is no reaction between the *M. phlei* antiserum and the other strains. In figure 5 the reactions are shown between the *M. ulcerans* strain and homologous antiserum and the failure of precipitation between *M. ulcerans* antiserum and the other cultures represented on the plate.

DISCUSSION

Rheins and associates (9) found it difficult to demonstrate antituberculous antibody in immune rabbit serum using a gel-diffusion method. Their results would suggest that this technique is considerably less effective for this purpose

than is indicated by the results reported in the present paper. There are several possible reasons for this difference. The results of unpublished experiments performed in this laboratory have shown that the intravenous injection of whole mycobacterial cells produced an antiserum in rabbits which was decidedly less effective for the detection of antigen by gel-diffusion methods than was the antiserum obtained when cell products or concentrated culture filtrates were incorporated in Freund's adjuvant and several injections given to rabbits subcutaneously. Whereas the former procedure produced antisera which apparently contained a low titer of a single antibody, the latter produced higher titer antisera which contained as many as six different antibodies.

The additional observation has been made that Old Tuberculin (OT), even in the concentrations used by Rheins and associates (9), functioned very poorly as an indicator antigen in double-diffusion reactions with either type of antisera. Using several concentrations of OT and PPD,[7] no more than one band of precipitate has been observed with any of the antisera noted in table 2. These results and, therefore, those of Rheins and associates are understandable when it is realized that considerable chemical manipulation is involved in the preparation of both OT and PPD. Such manipulations might easily affect the capacity of the antigenic substances to function as complete antigens.

Finally, the method employed by Rheins and co-workers of concentrating the antiserum by drying on paper discs may possibly have resulted in a loss of specific precipitating antibody due to denaturation.

The methods proposed in the present report circumvent the disadvantages mentioned above and provide more reactive rabbit antisera, with the end result that the gel-diffusion technique becomes capable of demonstrating as many as six different antigen-antibody complexes.

When a comparison is made between the groupings as originally reported (2) and the more recent arrangement as given in table 3, it is apparent that *M. phlei* now falls in a separate group, whereas formerly it had been placed with the other saprophytes. It is possible that in the earlier study contamination of either the cultures, the antigenizing filtrate preparation, or the antiserum with one of the *Group III* saprophytes or with *Group III* antiserum may have occurred. It is not likely, however, that the original culture was at fault, since cell suspensions of the original culture produced reactions identical to those obtained with the other 2 *M. phlei* strains obtained for the present investigation from Dr. Ruth Gordon. The more recent results with the *M. phlei* strains are probably correct, since three different cultures have been used and new rabbit antisera have been produced, using both the original *M. phlei* culture and the *M. phlei* strain No. 5.

The BCG-4 culture, originally placed with the *Group I* cultures, is now with the other bovine strains in *Group II*. This is explainable if one assumes that the BCG-4 culture may have been capable of greater antigen production in the liquid medium used in the earlier study than were the other bovine strains. If

[7] Manufactured by Parke, Davis and Company, Detroit, Michigan.

such was true, then a concentrated culture filtrate preparation from the BCG-4 strain would possess adequate concentrations of all of the 4 *Group II* antigens and therefore would produce four visible antigen-antibody complexes in subsequent precipitation plates. On the other hand, the 4 other bovine strains used in the earlier study may have been less efficient in this matter of antigen production and, therefore, may have failed to show the full complement of antigenic substances in the concentrated culture filtrate preparation.

The occurrence of the differences illustrated by the BCG cultures, together with the increased number of precipitate bands obtained with nearly all of the cultures, indicates that the use of viable cells as antigen sources does result in a more sensitive agar double-diffusion test than is possible when concentrated culture filtrate preparations are used.

Until the actual rates of antigen production by a bacterial strain can be predetermined, there is no way of anticipating the optimal time for taking readings on reaction plates, and, therefore, frequent observations of the plates are essential. Furthermore, due to the differences in the rate of antigen production by different strains, when more than one culture is present on a single plate one reaction may mature in advance of another, as is illustrated in figure 2. This merely constitutes an inconvenience since it is easily circumvented by daily observations of the reaction plates.

In attempting to decide the most satisfactory manner in which to group the 98 strains on the basis of the serologic data, the grouping given in table 4 appeared to be both logical and convenient. Theoretically, bacterial cultures should be antigenically grouped on the basis of antigens possessed in common by more than one species of an organism, viz., the streptococcal grouping antigens of Lancefield (8). From the present data there appears to be no one diffusible antigen common to all of the acid-fast organisms classified as mycobacteria. Lacking even one such commonly held antigen, the next most widely distributed antigens were used as the grouping antigens, hence the organisms grouped as *I*, *II*, *III*, *V*, and *VI*. The antigens responsible for the formation of *Groups IV*, *VII*, and *VIII* would appear to be more equivalent to the type-specific antigens of Lancefield, however, since all of the strains in each group represent single species. Actually, it is impossible to conclude whether the antigens detected by the agar double-diffusion precipitation reactions can be considered as group or type specific. For the purposes of uniformity and until other evidence to the contrary is obtained, it is more convenient, therefore, to consider them as grouping antigens. Following this same line of thought, perhaps it would be more exact to consider *Groups II* and *VI* as subgroups of *I* and *V*, respectively, but it seems at this early stage of development that such a procedure would actually serve little, if any, practical purpose.

It was of interest to note that there was no evidence of any antigenic relationship between any of the 98 mycobacterial strains studied and the 4 fungal cultures.

Finally, the so-called "atypical" acid-fast organisms do not appear to be

antigenically homogeneous, since they are represented in 5 of the 8 proposed serologic groups. Furthermore, there is no uniform pattern of distribution of photochromogenic, scotochromogenic, or nonphotochromogenic "atypical" mycobacteria within these five groups.

The close antigenic relationship of most of the "atypical" mycobacteria to the virulent mammalian and avian tubercle bacilli lends support to the possibility that there may be also a genetic relationship involved. The clear-cut lack of antigenic similarity between the "atypicals" and the saprophytic mycobacteria demonstrates that this simple double-diffusion method will provide an easy method for the differentiation between these two groups.

Summary

By means of an agar double-diffusion precipitation technique and using viable mycobacterial cell suspensions as a source of antigen, 98 strains of mycobacteria have been divided into eight antigenic groups.

Group I is comprised of 64 cultures. Nineteen of these cultures are typical strains of *M. tuberculosis* var. *hominis*, 42 are "atypical" acid-fast cultures, the remaining 3 are the avian strain Kirchberg and the strains *M. balnei* and vole. *Group II* is comprised of 5 "atypical" acid-fast cultures, 4 known bovine strains, and the avian strain Sheard. *Group III* contains some of the saprophytic mycobacteria and the strain *M. ranae*. *Group IV* consists of 3 strains of *M. phlei*. *Group V* is composed of 3 "atypical" acid-fast strains, a single recently isolated *M. tuberculosis* var. *hominis*, and *M. ulcerans*. *Group VI* contains only 2 "atypical" acid-fast strains. *Group VII* is similar to *Group IV* in that only a single species is represented, that is, three strains of *M. fortuitum*. Three "atypical" strains and *M. marinum* have been placed together in *Group* VIII. This is not a true group, however, as each culture formed a precipitate only with homologous antiserum.

Sumario

Relaciones Antigénicas entre Noventa y Ocho Cepas de Micobacterias Descubiertas con el Uso de Técnicas de Precipitación y Difusión de Gel

Por medio de una técnica de precipitación y doble difusión en agar y usando suspensiones de células micobacterianas viables para la obtención del antígeno, se dividieron 98 cepas de micobacterias en ocho grupos antigénicos.

El *Grupo I* está compuesto de 64 cultivos. Diecinueve de éstos son típicas cepas humanas, 42 son cultivos ácidorresistentes "atípicas" y los 3 restantes son la cepa aviaria Kirchberg y las cepas *balnei* y murina campestre. El *Grupo II* consta de 5 cultivos ácidorresistentes "atípicos", 4 cepas bovinas conocidas y la cepa aviaria Sheard. El *Grupo III* contiene algunas de las micobacterias saprofitas y la cepa *Ranae*. El *Grupo IV* consta de cepas del *M. phlei*. El *Grupo V* está compuesto de 3 cepas ácidorresistentes "atípicas", de un solo *M. tuberculosis* var. *hominis* recién aislado y del *M. ulcerans*. El *Grupo VI* contiene solamente 2 cepas ácidorresistentes "atípicas". El *Grupo VII* es semejante al *IV* en que sólo está representada una sola especie, o sean 3 sepas del *M. fortuitum*. En el *Grupo VIII* se han juntado 3 cepas "atípicas" y el *M. marinum*. Sin embargo, éste no constituye un verdadero grupo, pues cada cultivo formó un precipitado solamente con el antisuero homólogo.

Resume

Détermination des rapports antigéniques entre quatre-vingt-dix-huit souches de mycobactéries par les techniques de précipitation par diffusion en gelose

A l'aide de la technique de précipitation par double diffusion en gélose en utilisant comme antigènes des suspensions de cellules mycobactériennes viables, 98 souches de mycobactéries ont été divisées en huit groupes antigéniques.

Le *groupe I* comprend 64 cultures. Parmi celles-ci il y a 19 souches humaines types, 42 souches acido-résistantes "atypiques", plus 1 souche aviaire Kirchberg, 1 souche *Balnei* et 1 souche vole. Le *groupe II* comprend 5 cultures de souches acido-résistantes atypiques, 4 souches bovines identifiées et 1 souche aviaire Sheard. Le *groupe III* comprend certaines mycobactéries saprophytes et la souche *Ranae*. Le *groupe IV* comprend 3 souches de *M. phlei*. Le *groupe V* comprend 3 souches acido-résistantes "atypiques", 1 seule souche récemment isolée de *M. tuberculosis* var. *hominis* et de *M. ulcerans*. Le *groupe VI* ne comprend que 2 souches acido-résistantes "atypiques". Le *groupe VII* est semblable au *groupe IV* en ce sens qu'il n'est représenté que par une seule espèce, c'est-à-dire trois souches de *M. fortuitum*. Trois souches "atypiques" et *M. marinum* ont été réunies dans le *groupe VIII*. Celui-ci n'est pas un véritable groupe car chacune de ces cultures ne formait de précipité qu'avec le sérum homologue.

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THE DETECTION OF ANTIBODIES IN THE SERUM OF TUBERCULOUS PATIENTS BY AN AGAR DOUBLE-DIFFUSION PRECIPITATION TECHNIQUE[1, 2]

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INTRODUCTION

Rich recently (1) has reviewed the wealth of experimental work reported in the literature which has established that antibody may appear in the serum of tuberculous patients. Many attempts have been made to utilize this phenomenon for the diagnosis of active tuberculous disease in human beings. The methods which have been employed for the detection of antibody include the agglutination, complement-fixation, precipitation, and hemagglutination reactions. As has been pointed out by Topley and Wilson (2), however, none of these tests has proved to be sufficiently reliable or valid for the serologic diagnosis of tuberculosis.

The relatively recent development of gel diffusion precipitation techniques by Oudin (3) and Ouchterlony (4) suggested that one of these methods might be useful for the detection of antibody in the serum of patients with tuberculosis. The Oudin method, a simple diffusion technique, employs an agar column in which antiserum has been incorporated, and an antigen in solution is layered over this. During incubation, the antigens in the top layer will diffuse into the antibody-agar column and, when optimal concentrations of antibody to antigen occur, a separate band consisting of an insoluble precipitate of antigen and antibody will appear for each antigen-antibody system present.

The Ouchterlony technique is a modification of the Oudin method and differs from the latter in that both antigen and antibody are allowed to diffuse into a neutral agar medium. This modification has been designated as a double-diffusion method in contradistinction to the simple diffusion technique of Oudin.

The present paper gives the results obtained through the use of a modified double-diffusion precipitation method for the detection of antibodies against mycobacterial antibodies in the serum of clinically well persons and in the serum of persons suffering from tuberculosis and Hansen's disease.

MATERIALS AND METHODS

Antigens: The antigens used for analysis of human serum were unheated, concentrated Proskauer and Beck medium culture filtrates of the H37Rv strain of *M. tuberculosis*, the saprophytic 607 mycobacterial strain, *Nocardia pelliteriae*, *Penicillium notatum*, and

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Candida albicans cultures. These preparations were prepared in a manner similar to those culture filtrates used in a previous study by Parlett and Youmans (5).

Serum: The sera tested for the presence of precipitating antibody to *Mycobacterium tuberculosis* were obtained from three sources. Specimens of serum from 52 patients at Suburban Cook County Tuberculosis Sanatorium were obtained from both tuberculous as well as nontuberculous subjects, and each serum sample was given a number before delivery to this laboratory. The serologic analyses were completed before the results were correlated with the results of tuberculin tests, the clinical findings, and the final diagnoses.

The control batch of sera, which consisted of 38 specimens of serum from nontuberculous Mantoux-positive young adults, was obtained from medical students at Northwestern University Medical School.

A second group of 16 specimens of serum was obtained from Dr. Rola Wolcott at the U.S. Public Health Service Hospital, Carville, Louisiana, and consisted of serum from 13 patients known to have Hansen's disease and serum from 3 healthy workers at the hospital.

Double-diffusion precipitation technique: The method developed for testing human serum for the presence of tuberculous antibody was a modification of the tube double-diffusion technique described by Oakley (6) and was similar to the technique used by Seibert and Figueroa (7) in gel-diffusion studies of tuberculin proteins and polysaccharides. Pyrex® tubes 10 cm. long and with an inside diameter of 3 mm. were prepared from glass tubing. These tubes were chemically cleaned, rinsed well with double distilled water, and inverted in a beaker which contained sufficient 0.1 per cent melted Difco purified agar to cover 3 to 4 cm. of the tubes. The whole was then autoclaved and the tubes were subsequently drained of excess agar and dried overnight in a 50°C. hot air oven. The interior of each tube was thus coated with an adherent film of dried agar which prevented the separation of the subsequently added agar column from the wall of the tube.

The various antigen preparations were diluted in a ratio of two parts of antigen to one part of 1.0 per cent Difco purified agar which had been prepared with 0.9 per cent sodium chloride and cooled to 48°C. The tubes were filled with a capillary pipette to one-third volume with this antigen agar solution and allowed to stand until the solution jelled. A second layer of 0.5 per cent saline agar was added to the first layer until two-thirds of the total tube capacity had been filled. Finally, after the second layer was solidified, the tube was filled with undiluted serum to be tested.

The tubes were then placed in clean, sealed mason jars and incubated at 37°C. for a maximal period of three weeks. Daily observations were made. In most instances precipitation was apparent within forty-eight to seventy-two hours and was maximal within five days. Rarely did bands of precipitate develop after five days.

When unmistakably sharp bands of precipitate were observed in the tubes, the tests were considered positive. The absence of true antigen-antibody precipitate was interpreted as indicating the absence of detectable tuberculous antibodies in the patient's serum. Typical positive and negative reactions are illustrated in figure 1. The density and size of the precipitate bands are much greater when viewed directly using transmitted light than they appear to be in the photograph shown in figure 1.

In practice, the tubes were cleaned, coated, filled with the antigen layer, and stored at 4°C. for as long as four weeks prior to use in the tests without evidence of decreased sensitivity, provided they were stored in a closed container which prevented evaporation. Contamination of these stored tubes seldom was observed, probably because of the small diameter of the exposed surface, the depth of the tube, and the paucity of oxygen in the agar column. Immediately prior to use, the second layer of neutral agar was overlayered and the serum to be tested was added.

Results

The results presented in the tables were obtained from a minimum of two assays per serum. In table 1 detailed results and related clinical information are

TABLE 1

SERUM FROM THIRTY-SEVEN TUBERCULOUS PERSONS WHICH PRECIPITATED ONE OR MORE ANTIGENS FROM A CULTURE FILTRATE PREPARATION OF H37Rv IN DOUBLE-DIFFUSION PRECIPITATION TUBES

Case Number	Results of Tuberculin Test*	Clinical and Laboratory Data
1		No present evidence of active tuberculosis; nephrectomy for tuberculous kidney in 1956
32	8 mm. PPD-S	
40	3 mm. PPD-S	
42	1 mm. PPD-S	
43	Negative to PPD-S and OT 1:10	
51	13 mm. PPD-S	
52	3 mm. PPD-S	
53	12 mm. PPD-S	
54	10 mm. PPD-S	
56	11 mm. PPD-S	
57	15 mm. PPD-S	
58	Negative to PPD-S and OT 1:10	Bacteriologic proof of active pulmonary tuberculosis obtained
61	10 mm. PPD-S	
66	Positive to PPD (second-strength)	
67	Not tested	
68	Not tested	
70	Not tested	
72	Not tested	
83	Positive to 1:1,000 OT	
76	Not tested	
93	Not tested	
87	Not tested	
85	Not tested	
59	4 mm. PPD-S	Typical *M. tuberculosis* from sputum; silicotuberculosis
45	13 mm. PPD-S	Typical *M. tuberculosis* from empyema fluid
48	8 mm. PPD-S	Typical *M. tuberculosis* from pleural effusion fluid culture
62	Positive to 1:10,000 OT	Sputum culture negative for *M. tuberculosis;* one sputum smear positive for *M. tuberculosis;* clinical symptoms present
65	Positive to 1:1,000 OT	Typical *M. tuberculosis* cultured from sputum; pneumonectomy
71	Not tested	Pott's disease, 15-year history; paravertebral abscess
41	6 mm. PPD-S	Tuberculous endobronchitis
69	Positive to 1:1,000 OT	Sputum cultures negative for *M. tuberculosis;* roentgenograms compatible with minimal tuberculosis; wife of patient with chronic disease
73	Positive to 1:1,000 OT	Typical *M. tuberculosis* cultured from pleural effusion

* 0.00002 mg. PPD-S employed. Tests are recorded in millimeters of induration at forty-eight hours. Data using PPD-S provided through the courtesy of Dr. Lydia Edwards, U. S. PHS.

TABLE 1 (*Continued*)

Case Number	Results of Tuberculin Test*	Clinical and Laboratory Data
55	15 mm. PPD-S	Old history of chronic disease; sputum cultures currently negative for *M. tuberculosis*
21	19 mm. PPD-S	Typical *M. tuberculosis* cultured from sputum post pneumonectomy (1956)
44	12 mm. PPD-S	Pre-pneumonectomy sputum culture positive for *M. tuberculosis;* currently negative
49	11 mm. PPD-S	Pre-pneumonectomy sputum culture positive for *M. tuberculosis;* currently negative
60	12 mm. PPD-S	Pre-pneumonectomy sputum culture positive for *M. tuberculosis;* currently negative

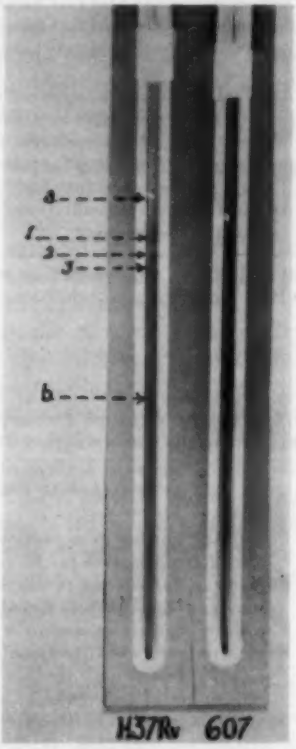

FIG. 1. Typical positive reaction between serum from a tuberculous patient and H37Rv antigens, and the lack of precipitation with the 607 culture filtrate preparation: *a* designates the meniscus between antiserum and neutral agar, *b* is the meniscus between the antigen layer in the bottom of the tube and the neutral agar layer. The numbers 1, 2, and 3 identify three bands of precipitate which appeared in the H37Rv reaction tube only. Actual size.

TABLE 2

SERUM FROM TWELVE APPARENTLY TUBERCULOUS PERSONS FROM WHOM ATYPICAL ACID-FAST ORGANISMS WERE CULTURED AND WHICH PRECIPITATED ONE OR MORE ANTIGENS FROM A CULTURE FILTRATE PREPARATION OF H37Rv IN DOUBLE-DIFFUSION PRECIPITATION TUBES

Case Number	Result of Tuberculin Test*	Characteristi s of Isolated Culture
20	6 mm. PPD-S	Scotochromogenic acid-fast bacilli from case of pulmonary fibrosis
25	13 mm. PPD-S	Scotochromogenic acid-fast bacilli from sputum
29	Positive to 1:1,000 OT	Photochromogenic acid-fast bacilli from sputum
46	2 mm. PPD-S	Photochromogenic acid-fast bacilli from sputum
64	Not tested	Scotochromogenic acid-fast bacilli from sputum
4	7 mm. PPD-S	Scotochromogenic acid-fast bacilli from sputum
30	12 mm. PPD-S	Scotochromogenic acid-fast bacilli from sputum
89	Positive to 1:1,000 OT	Scotochromogenic acid-fast bacilli from sputum
38	16 mm. PPD-S	Atypical acid-fast organism cultured from sputum
28	14 mm. PPD-S	Sputum culture negative for tubercle bacilli post pneumonectomy (1956); scotochromogen isolated
36	Positive to 1:1,000 OT	Sputum culture negative for tubercle bacilli post pneumonectomy; atypical acid-fast bacilli from surgical specimen
27	3 mm. PPD-S	Atypical acid-fast bacilli from surgical specimen obtained through thoracoplasty

* 0.00002 mg. PPD-S employed. Tests are recorded in millimeters of induration at forty-eight hours. Data using PPD-S provided through the courtesy of Dr. Lydia Edwards U. S. PHS.

TABLE 3

SERUM FROM FOUR PATIENTS WITH CHRONIC PULMONARY DISEASE WHICH FAILED TO PRECIPITATE ANTIGENS IN ANY OF THE CULTURE FILTRATE PREPARATIONS USED IN DOUBLE-DIFFUSION PRECIPITATION TUBES

Case Number	Results of Tuberculin Test*	Clinical Impression
47	8 mm. PPD-S	Pulmonary fibrosis in a previously employed chalk cutter; lung biopsy negative for *M. tuberculosis*
16	3 mm. PPD-S	Pulmonary disease due to atypical acid-fast bacilli isolated from sputum. This was a scotochromogenic culture which was serologically related to *M. ulcerans*.
50	2 mm. PPD-S	Student nurse; one questionable sputum smear with repeatedly "negative" sputum cultures; no clinical symptoms of active tuberculosis
63	Positive to 1:1,000 OT	Sputum cultures consistently "negative"; chronic cough without other clinical evidence of tuberculosis

* 0.00002 mg. PPD-S employed. Tests are recorded in millimeters of induration at forty-eight hours. Data using PPD-S provided through the courtesy of Dr. Lydia Edwards, U. S. PHS.

given for 37 specimens of serum obtained from tuberculous individuals. All of these sera precipitated one or more of the antigens known to be present in the H37Rv culture filtrate preparation and failed to precipitate any of the control antigens. As is indicated also in table 1, evidence of active pulmonary tuberculosis other than serologic also was obtained for each of the 37 patients. Similar reactions were obtained with serum of 12 patients reported in table 2, except that the microorganisms isolated bacteriologically were atypical acid-fast organisms.

Of the total of 52 patients in Suburban Cook County Tuberculosis Sanatorium whose serum was analyzed by the tube double-diffusion technique, the serum of only 4 failed to show the presence of antibodies for any of the antigens. All information pertaining to these 4 serum specimens is given in table 3. The serum from *Case 16*, however, gave a positive reaction in a separate test with a culture filtrate antigen prepared from a culture of *Mycobacterium ulcerans* and also with an antigen prepared from the culture isolated from the patient's sputum. These tests were performed because, in another study, the mycobacterium isolated from the sputum of this patient had been found to react only with an antiserum prepared against *M. ulcerans*. This finding is consistent with results obtained in unpublished experiments which demonstrate that *Mycobacterium ulcerans* and *Mycobacterium tuberculosis* var. *hominis* are antigenically different. The remaining 3 specimens of serum shown in table 3 were obtained from patients who were proved not to have tuberculosis.

The 16 specimens of serum (13 from patients with Hansen's disease and 3 from normal persons) obtained from the U. S. Public Health Hospital in Carville, Louisiana, contained no detectable antibody.

Thirty-six of 38 specimens of serum from medical student volunteers at Northwestern University Medical School who were Mantoux positive when tested by the intradermal injection of either a 1:5,000 or a 1:100 dilution of Old Tuberculin (OT) failed to precipitate H37Rv or control concentrated culture filtrate antigens in the double-diffusion tubes. Two of the 38 specimens were observed to give strong precipitating reactions with the H37Rv antigen. One of the students had had a nephrectomy (*Case 1*, table 1) because of a tuberculous kidney eleven months prior to the donation of this serum, and the second subject was a graduate student engaged in research which required working with cultures of virulent tubercle bacilli. Roentgenographic chest films, however, to date have revealed no evidence of an active tuberculous process.

Discussion

The results of serologic tests performed for the purpose of providing information on the presence or absence of an active infectious process are difficult to interpret. In the first place, an antibody found in the serum of a human being which is specific for an antigen produced by, or present in, some pathogenic microorganism may indicate either the presence of active disease; a previous infection from which the person has recovered; a previous vaccination; or the

presence of a latent, inactive, or subclinical infection. In addition, the wide variation in the ability of human beings to produce antibodies in response to an antigenic stimulus means that little reliance can be placed on single determinations of the antibody content of the serum. The amount of antibody in the serum also may be affected by the continuous production of antigens by microorganisms in the tissues. Finally, attempts to increase the sensitivity of techniques for the detection of antibody in the serum have led to the development of methods which rely upon reagents and indicator systems of such inherent instability that both the specificity and reliability of the tests may be altered significantly. In tuberculosis, the failure to appreciate the possible influence of the above-mentioned factors has led in turn to the adoption and then to the abandonment of such serologic diagnostic tests as complement-fixation and hemagglutination.

In the serologic diagnosis of many of the more acute infectious diseases, an increase in the amount of antibody in the serum over a period of a few days is generally regarded as being highly indicative of an active infection. This reasoning is a logical consequence of the fact that, regardless of the total amount of antibody a human being is capable of forming, the amount produced will increase up to a certain maximum in response to the antigenic stimulus furnished by the presence of the invading parasite. In a chronic disease such as tuberculosis, however, the antibody level may have reached a maximum by the time clinical or roentgenographic evidence of active disease is obtained. Moreover, the antibody content of the serum may fluctuate because of an irregular production of antigen by the infecting bacilli, or the amount of antibody may be low because of an overabundance of antigen in the tissues. Furthermore, some degree of immunologic paralysis may be brought about by the continuous production of antigen by the persistently present viable microbes. Present knowledge of the influence of these factors on the serum antibody level in tuberculosis is very limited. Therefore, a change in the antibody content of the serum may not have the same degree of significance which can be attached to similar changes which occur in some of the more acute infectious diseases. This further complicates the possible usefulness of serologic diagnostic tests in tuberculosis.

At present there is a great need for a simple, reliable technique which will measure with a high degree of specificity the individual antibodies against *Mycobacterium tuberculosis* which may appear in the serum of human beings. With such a test, information could be obtained which would reveal the relationship between active tuberculous disease, or tuberculous infection, and the rise and fall in the amounts of the antibodies in the serum.

The tube double-diffusion precipitation method used to obtain the results reported in this paper possesses certain advantages for this purpose. It is a simple and reliable technique, and the determinations can be made in hospital laboratories possessing a minimum of equipment. It is also a highly specific test in the sense that only serum containing antibody against antigens in the antigenic preparation employed will react. In other words, nonspecific reactions due to reagents used in the tests or due to technical factors, so common in the complement-fixation and hemagglutination tests, do not occur. This is the result of the

fact that the antigen-antibody complex formed is observed directly. Unstable reagents and complicated indicator systems are not required. Also the test is far more sensitive than the conventional tube precipitation reaction, since at least six different antibodies can be enumerated clearly and separately. Furthermore, judging from the results reported in this paper, there is a possibility that the method may be a specific test for tuberculosis. The negative results obtained with the serum of patients with Hansen's disease are of particular significance in this respect. Finally, the negative results obtained with the serum of the tuberculin-positive, clinically well medical students may be of significance. If it is assumed that at least some of the students had latent tuberculous infections, these findings strongly indicate that the test may be of some usefulness in distinguishing between latent tuberculous infection and active tuberculous disease. Much more data of a similar nature will have to be acquired, however, before such a conclusion can be made validly.

On the negative side, attention must be called to the fact that the double-diffusion precipitation method as used in this study is purely qualitative in nature. It provides no measurement of the amount of any of the antibodies detected. It does provide the opportunity, however, to enumerate the number of specific precipitating mycobacterial antibodies present in the serum. In this connection it is of interest to report that serial blood samples spaced one month apart were obtained from a single patient with tuberculosis while the patient was receiving therapy. The samples were then analyzed by the double-diffusion technique. It was observed that the number of precipitate bands had decreased, and the amount of precipitate in the remaining bands was less in those serum samples obtained one and two months after the sputum cultures became negative for tubercle bacilli. If such a decrease in number and intensity of bands is an indication of a decrease in antibody content, then the tube double-diffusion test may possess quantitative potentialities. It will be necessary to analyze a large number of such serial samples, however, before its value in this respect will be known.

The data reported on *Case 16* in table 3 are of particular interest. In this case no antibodies were found in the serum with any of the culture filtrate antigens employed routinely. When antigens were used which had been prepared from filtrates of cultures of *Mycobacterium ulcerans* and from cultures of the organism isolated from the sputum of the patient of *Case 16*, several bands of precipitate were noted. In other investigations in this laboratory, in which a modified Ouchterlony double-diffusion precipitation technique was employed, it has been found that mycobacteria can be arranged into a number of antigenically distinguishable groups (5, 8). In the present studies it was found that the culture isolated from *Case 16* fell into the antigenic group of which *Mycobacterium ulcerans* was the type strain. In addition to the culture obtained from *Case 16*, five other atypical bacilli which show the same relationship to *Mycobacterium ulcerans* have been isolated from patients with pulmonary disease. To date, however, no serum has been obtained from these patients. Although most atypical

mycobacteria are antigenically related to mammalian tubercle bacilli (8), the finding obtained with the serum from *Case 16* demonstrates that, by means of the double-diffusion precipitation technique, a closer relationship sometimes can be demonstrated between certain antigenically distinct atypical acid-fast bacilli isolated from the sputum and the pulmonary disease present in the patient.

Burrell and associates (9) have studied the character and distribution of tuberculous antibody in human serum by a gel-diffusion technique. Their results were disappointing since only 5 (12.5 per cent) of 40 specimens of tuberculous serum tested showed the presence of tuberculous antibody. It is possible that this percentage would have been higher, however, had an antigen other than OT been used. In this laboratory it has been found that OT functions very poorly as a detector of precipitating tuberculous antibody in gel-diffusion systems (8).

Summary

Serum obtained from 53 patients was tested for the presence of circulating antibody by means of a tube double-diffusion precipitation method. The antigens employed were concentrated filtrate preparations of cultures of *Mycobacterium tuberculosis* var. *hominis*, strain H37Rv, the saprophytic mycobacterial strain 607, *Nocardia pelliteriae*, *Penicillium notatum*, and *Candida albicans*. All but 4 of the 53 specimens of serum possessed antibodies which reacted only with the antigens present in the concentrated culture filtrate of the H37Rv culture of *Mycobacterium tuberculosis* var. *hominis*. All of the 48 patients whose serum had been found to contain tuberculous antibody had evidence of tuberculous disease. On the other hand, of the 4 specimens of serum which were negative in this test, 3 came from subjects who did not have tuberculous disease and one came from a patient with pulmonary disease caused by an atypical acid-fast microorganism which was antigenically related to *Mycobacterium ulcerans*. Antibodies specific for *M. ulcerans* were found in the serum of this patient.

Serum was also obtained from 38 clinically well persons who at the time the blood samples were obtained were found to give a positive reaction following the intradermal injection of either 1:5,000 or 1:100 dilution of OT. Only two of the specimens from the 38 healthy persons were found to contain antibodies against mycobacterial antigens. One of the two whose serum gave a positive test had had a tuberculous kidney removed eleven months previously. The other individual was a graduate student who had been working with cultures of virulent tubercle bacilli in the laboratory for a period of several months. No evidence of active tuberculous disease, however, has been noted in this person.

Sera from 13 patients with Hansen's disease were tested, using the same antigen preparations. None of these sera reacted with any of the antigenic preparations employed.

Sumario

El Descubrimiento de Anticuerpos en los Sueros de Tuberculosos con una Técnica de Precipitación y Doble Difusión en Agar

Sueros obtenidos de 53 enfermos fueron comprobados en cuanto a presencia de anticuerpos por medio de un método de precipitación y doble difusión en tubo. Los antígenos

empleados fueron preparaciones filtradas concentradas de cultivos del *Mycobacterium tuberculosis* var. *hominis*, cepa H37Rv, la cepa micobacteriana saprofita 607, *Nocardia pelliteriae*, *Penicillium notatum* y *Candida albicans*. Aparte de 4, todos los 53 sueros poseían anticuerpos que no reaccionaban más que con los antígenos presentes en el filtrado cultural concentrado de la cepa H37Rv del *M. tuberculosis* var. *hominis*. Todos los 48 enfermos en cuyos sueros se encontraron anticuerpos tuberculosos tenían signos de enfermedad tuberculosa. En cambio, del los 4 sueros que resultaron negativos en esta comprobación, 3 procedían de sujetos que no tenían afección tuberculosa y uno de un sujeto que tenía enfermedad tuberculosa producida por un microbio ácidorresistente atípico que estaba relacionado antigénicamente con el *Mycobacterium ulcerans*. En el suero de este enfermo se descubrieron anticuerpos específicos para el *M. ulcerans*.

Se obtuvieron además sueros de 38 personas clínicamente sanas que, en la fecha en que se obtuvieron las muestras de sangre, mostraron una reacción positiva a continuación de la inyección intradérmica de diluciones al 1:5,000 ó 1:100 de tuberculina antigua. Sólo en 2 de los sueros de las 38 personas sanas se descubrieron anticuerpos contra los antígenos micobacterianos. A uno de los 2 cuyos sueros acusaron reacciones positivas le habían extirpado un riñón tuberculoso once meses antes; el otro era un estudiante recibido que había estado trabajando con bacilos tuberculosos virulentos en el laboratorio durante un período de varios meses, aunque nunca se habían notado en él signos de afección tuberculosa.

Los sueros de 13 sujetos que padecían de la enfermedad de Hansen fueron comprobados con las mismas preparaciones de antígenos. Ninguno de estos sueros reaccionó con ninguna de las preparaciones antigénicas empleadas.

Resume

La détection des anticorps dans le sérum des tuberculeux par la méthode de précipitation par double diffusion dans la gélose

Des sérums prélevés chez 53 malades furent testés pour la recherche d'anticorps au moyen de la méthode de précipitation par double diffusion en tubes de Pyrex. Les antigènes utilisés étaient des préparations de filtrats concentrés de cultures de *Mycobacterium tuberculosis* var. *hominis*, souche H37Rv, de souche mycobactérienne saprophyte 607, de *Nocardia pelliteriae*, de *Penicillium notatum* et de *Candida albicans*. Les sérums, sauf 4 sur 53 possédaient des anticorps ne réagissant qu'aux antigènes présents dans les filtrats de culture de la souche H37Rv du *M. tuberculosis* var *hominis*. La totalité des 48 malades, dont le sérum renfermait les anticorps tuberculeux présentaient des signes de tuberculose. D'autre part, parmi les 4 sérums où ce test était négatif, 3 provenaient de sujets non tuberculeux et l'autre provenait d'un malade atteint d'une affection pulmonaire due à un germe acido-résistant atypique apparenté antigéniquement au *Mycobacterium ulcerans*. Le sérum du malade contenait des anticorps spécifiques pour le *M. ulcerans*.

Les sérums furent également prélevés chez 38 sujets cliniquement bien portants; toutefois, au moment où le prélèvement fut effectué, ils présentaient une réaction positive à l'inoculation intradermique de tuberculine de Koch en dilution à 1:5000 ou 1:100. Parmi les sérums de ces 38 sujets bien portants, 2 sérums seulement possédaient des anticorps contre les antigènes mycobactériens. Des deux sujets dont le sérum avait donné une réaction positive, l'un avait subi onze mois auparavant l'ablation d'un rein tuberculeux, l'autre provenait d'un chercheur qui pendant une période de plusieurs mois faisait des recherches au laboratoire sur des cultures de bacilles tuberculeux virulents. Aucun signe de tuberculose en activité n'a cependant été décelé chez ce sujet.

Les sérums de 13 lépreux ont été testés avec ces mêmes préparations antigéniques. Aucun d'eux n'a réagit à l'une ou l'autre de ces préparations antigéniques.

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PERSISTENCE OF TUBERCLE BACILLI IN THE ORGANS OF GUINEA PIGS UNDER CHEMOTHERAPY[1]

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INTRODUCTION

It has been shown that streptomycin has little or no effect in the early phases of experimental tuberculosis. Thus, when guinea pigs are infected with virulent tubercle bacilli and treatment with streptomycin is started a few days before the infection or on the day of infection, dissemination of bacilli and production of lesions are not influenced at all, at least during the first twenty-one days of treatment (1). In mice also, it is not possible to eliminate tubercle bacilli with the chemotherapeutic agents currently used. For example, isoniazid will first reduce the number of bacilli in the spleens of these animals, although later on the number is stabilized regardless of how long the treatment is prolonged, and bacilli will always be present in several organs (2, 3).

Humbert (4) treated guinea pigs for fourteen months with isoniazid and four months later could not detect any lesions; he concluded, however, that despite the negative histologic findings the tissues remained infected. Vaccarezza and collaborators (5) and Sher and associates (6) stated that guinea pigs treated with isoniazid were by no means completely sterilized with respect to tubercle bacilli. Ferebee and Palmer (7) noticed that fourteen weeks after withdrawal of isoniazid, infected guinea pigs have the same survival rate as the noninfected controls. Bartmann and associates (8) carried out similar experiments although their conclusions were somewhat different.

The present paper is concerned with the combined therapeutic effect of streptomycin and isoniazid on guinea pigs infected with tubercle bacilli. The treatment in this study was given for various periods of time and the animals were observed for prolonged periods.

MATERIAL AND METHODS

Three groups of tuberculin-negative guinea pigs weighing 350 to 450 gm. were used, 19 in *Group A*, 31 in *Group B*, and 31 in *Group C*. Each animal was given a subcutaneous inoculum of 0.2 mg. moist weight of tubercle bacilli in the anterior aspect of the left thigh. The suspension used was prepared with the H37Rv[2] strain of *Mycobacterium tuberculosis* var. *hominis*, grown for 21 days in Löwenstein-Jensen medium, the bacterial density being determined in Hopkins tubes.

Seven animals from each one of the three groups were used as controls of the infection. The remainder were treated with intramuscular injections of 6 mg. of streptomycin three times a week and with 5 mg. of isoniazid orally per day. Treatment was started the day of the infection and it was continued for 15 days in the 12 animals of *Group A*, for 30 days in

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[2] From the American Type Culture Collection # 9360.

the 24 animals of *Group B*, and for 60 days in the 24 animals of *Group C*. Subsequent procedures varied for each group and are described separately.

Treated and subinoculated animals as well as the infected controls were housed separately, one guinea pig per cage.

Group A: At the end of 15 days of treatment, the 7 control (untreated) animals were sacrificed, as well as 6 of the 12 experimental (treated) animals. Fragments of regional lymph nodes, spleen, liver, and lungs were taken from 5 of the 6 sacrificed (treated) animals. Each one of these fragments was separately macerated in sterile mortars, and one guinea pig was subinoculated with each fragment in the subcutaneous tissue of the anterior aspect of the left thigh. Thus, the total number of subinoculated guinea pigs was 20. These subinoculated animals were tested with tuberculin approximately every month and when the skin reaction became positive, they were sacrificed.

The other 6 experimental (treated) guinea pigs were sacrificed 39 days after treatment was discontinued (or 54 days after inoculation).

Group B: The procedure followed in this group was similar to that in *Group A* except that treatment was prolonged for 30 days instead of 15. Thus, the 7 control (untreated) animals were sacrificed 30 days after inoculation, together with 9 experimental (treated) animals. Fragments of regional lymph nodes, spleen, liver, and lungs were taken from 5 of the 9 experimental (treated) animals which were sacrificed. Each one of these fragments was treated in the fashion described above, so 20 healthy guinea pigs were subinoculated and sacrificed when the tuberculin test became positive.

The other 15 experimental (treated) animals were divided into two groups: 8 were observed for 60 days and sacrificed and 7 were observed for 90 days and sacrificed.

Group C: The 31 animals in this group were treated for 60 days. Seven control (untreated) and 9 experimental (treated) animals were sacrificed at the end of this period; macerates of the organs from 5 of the 9 sacrificed (treated) animals were subinoculated into 20 healthy guinea pigs as described before. These 20 animals were tested with tuberculin approximately every month, and when the test became positive they were sacrificed.

The remaining 15 experimental (treated) animals were again divided in two groups: 8 were observed for 60 and 7 for 240 days respectively, after treatment was stopped, and were then sacrificed also.

Bacteriologic and pathologic studies: All guinea pigs were examined at autopsy, and in almost all (exceptions will be noted) touch smears, cultures in Löwenstein-Jensen medium, and hematoxylin-eosin sections of lymph node, spleen, liver, and lungs were made. Drug-susceptibility studies to streptomycin and to isoniazid were also performed on all positive cultures except those from control animals.

The methods of study described in the preceding paragraphs are summarized in table 1.

Results

Control animals: The lesions present in the control animals sacrificed 15, 30 and 60 days after inoculation were more numerous and apparent, the longer the infection was allowed to progress.

Group A animals: With one exception, it was not possible to observe gross lesions in the lymph nodes, spleen, liver, and lungs of the animals treated with isoniazid-streptomycin for 15 days. The exception was one lesion in the iliac lymph node of one guinea pig. Smears or cultures of these organs were positive for tubercle bacilli in one-third of the animals, especially in material from the iliac lymph node.

The histologic study of the tissues showed many more lesions than were suspected from the gross aspect. Nevertheless, the lesions were not as numerous as in the control animals, and the lesions were absent in the liver and scarce in the lungs (table 2).

TABLE 1

OUTLINE OF THE METHOD FOLLOWED IN THE PRESENT STUDY

Group A 19 guinea pigs	7 controls sacrificed 15 days after inoculation			
	12 experimental ones, treated with SM-INH for 15 days	6 sacrificed right after completion of treatment	Macerates of each of several organs taken from 5 guinea pigs were used for subinoculation of 20 healthy guinea pigs	Sacrificed 140 days after subinoculation, having a positive tuberculin test
		6 sacrificed 39 days after completion of treatment		
Group B 31 guinea pigs	7 controls sacrificed 30 days after inoculation	9 sacrificed right after completion of treatment	Macerates of each of several organs taken from 5 guinea pigs were used for subinoculation of 20 healthy guinea pigs	6 sacrificed after 150 days and 14† sacrificed after 300 days, having a positive tuberculin test
	24 experimental ones, treated with SM-INH for 30 days	8 sacrificed 60 days after completion of treatment		
		7 sacrificed 90 days after completion of treatment*		
Group C 31 guinea pigs	7 controls sacrificed 60 days after inoculation	9 sacrificed right after completion of treatment	Macerates of each of several organs taken from 5 guinea pigs were used for subinoculation of 20 healthy guinea pigs	Sacrificed 240 days after subinoculation, having a positive tuberculin test
	24 experimental ones, treated with SM-INH for 60 days	8 sacrificed 60 days after completion of treatment		
		7 sacrificed 240 days after completion of treatment		

* No histologic studies were carried out in this group.

† Tuberculin test turned positive after 200 days, but the animals were sacrificed 300 days after subinoculation.

SM: streptomycin; INH: isoniazid.

Smears, cultures and histologic studies were performed on the majority of the animals.

TABLE 2

HISTOPATHOLOGIC STUDY OF GROUP A

Subgroups	Microscopic Lesions			
	Regional Lymph Nodes	Spleen	Liver	Lungs
Controls	Granulomas with scarce caseous necrosis and polymorphonuclear leukocytes	Granulomas with scarce necrosis	Granulomas with scarce necrosis	Scarce granulomas without necrosis
Treated for 15 days	Granulomatous and/or reticular cell hyperplasia	Scarce granulomas	No lesions	Scarce granulomas
Treated for 15 days and observed for another 39 days after completion of treatment	Extensive granulomas with much necrosis	Granulomas with necrosis	Granulomas without necrosis	Scarce granulomas without necrosis

TABLE 3

HISTOPATHOLOGIC STUDY OF ANIMALS SUBINOCULATED WITH MACERATES OF ORGANS FROM GUINEA PIGS OF GROUP A, TREATED FOR 15 DAYS*

Organ Used for Inoculation	Microscopic Lesions			
	Regional Lymph Nodes	Spleen	Liver	Lungs
Regional lymph node	Granulomas with caseous necrosis	Granulomas without necrosis	Granulomas	Granulomas
Spleen	Granulomas with scarce necrosis	Granulomas with extensive necrosis	Granulomas	Granulomas
Liver	Granulomas with scarce necrosis	Scarce granulomas	No lesions	No lesions
Lung	Granulomas with caseous necrosis	Scarce granulomas	No lesions	No lesions

* These animals were sacrificed 140 days after subinoculation.

The presence of viable bacilli in the organs of guinea pigs treated with isoniazid-streptomycin for 15 days was demonstrated by the fact that the animals subinoculated with macerates of these organs became tuberculin positive after 140 days. Moreover, the subinoculated animals showed both gross and microscopic tuberculous lesions in many organs at autopsy (table 3). It seems likely

TABLE 4

HISTOPATHOLOGIC STUDY OF GROUP B

Subgroups	Microscopic Lesions			
	Regional Lymph Nodes	Spleen	Liver	Lungs
Controls	Granulomas with necrosis and epithelioid cells	Granulomas with necrosis	Granulomas with scarce necrosis	Granulomas with giant cells
Treated for 30 days	No lesions	No lesions	No lesions	Scarce granulomas
Treated for 30 days and observed for another 30 days after completion of treatment	Granulomas	Reticular hyperplasia	No lesions	Granulomas

Another group was sacrificed 90 days after completion of treatment but no histopathologic studies were done.

that there were more tubercle bacilli in the spleen and lymph nodes than in the liver and lungs, since the animals subinoculated with the former organs showed more lesions than those subinoculated with the latter tissues.

Tuberculous lesions reappeared in animals after the treatment was discontinued, as it was shown by the 6 guinea pigs sacrificed 39 days after chemotherapy was stopped. The histologic study revealed more lesions than those which were apparent grossly.

Group B animals: After 30 days of treatment with isoniazid-streptomycin, it was difficult to observe either gross or microscopic lesions and, when such lesions were found, they were scarce and unimportant (table 4). No tubercle bacilli were found either by smear or culture. However, when treatment was discontinued and 30 days more were allowed for observation, some of the animals showed a few microscopic lesions. In these circumstances, smears from the site of inoculation were positive for tubercle bacilli in 2 cases, and tubercle bacilli were cultured from that area in 3 cases. If instead of allowing 30 days for post-treatment observation, 90 days were permitted, the lesions became grossly visible in the iliac lymph node. The presence of viable bacilli was demonstrated by subinoculation of the organs of animals sacrificed after 30 days of treatment. As in *Group A*, guinea pigs subinoculated with suspensions of spleen showed more lesions than those subinoculated with other organs (table 5).

Group C animals: When the isoniazid-streptomycin treatment was prolonged for 60 days, there were only a few microscopic lesions in some animals, and these were always of the fibrotic, regressive type (table 6). Smears and cultures were also negative for tubercle bacilli. Furthermore, if the animals were observed for 60 days after completion of the treatment, the results were the same. Lesions

TABLE 5

HISTOPATHOLOGIC STUDY OF ANIMALS SUBINOCULATED WITH MACERATES OF ORGANS FROM GUINEA PIGS OF GROUP B, TREATED FOR 30 DAYS*

Organ Used for Inoculation	Microscopic Lesions			
	Regional Lymph Nodes	Spleen	Liver	Lungs
Regional lymph node	Few granulomas, except in 2 animals	No lesions	No lesions	No lesions
Spleen	Granulomas with necrosis	Granulomas with minimal necrosis	Scarce portal granulomas without necrosis	Extensive granulomas with giant cells
Liver	No lesions	No lesions	No lesions	Epithelioid cells and polymorphonuclear leukocytes in one-half of the total of animals
Lung	No lesions	No lesions	No lesions	No lesions

* Six animals of this group were sacrificed 150 days after subinoculation and the remaining 14 after 300 days, all with clearly positive tuberculin reactions.

TABLE 6

HISTOPATHOLOGIC STUDY OF GROUP C

Subgroups		Microscopic Lesions			
		Regional Lymph Nodes	Spleen	Liver	Lungs
Controls		Extensive granulomas with caseous necrosis	Granulomas with extensive necrosis	Granulomas	Granulomas with caseous necrosis
Treated for 60 days		No lesions	No lesions	No lesions	No lesions
Treated for 60 days and	Sacrificed 60 days after completion of treatment	No lesions	No lesions	Perivascular scars	Perivascular scars
	Sacrificed 240 days after completion of treatment	Extensive granulomas with caseous necrosis	No lesions	No lesions	No lesions

appeared only after 240 days of observation after treatment and were limited to the iliac lymph nodes. Smears and cultures were positive for tubercle bacilli only in these lymph nodes. The animals subinoculated with suspensions of the organs of guinea pigs treated for 60 days were sacrificed after 240 days of observation, when they gave positive tuberculin reactions; nevertheless, no gross or microscopic lesions were discovered in this group, and cultures of all organs were negative for tubercle bacilli.

All strains isolated were tested for susceptibility to isoniazid and streptomycin and they were all drug susceptible.

Discussion

The results described in the experimental part of this paper raise a number of interesting questions related to both the effect of isoniazid-streptomycin treatment on different phases of experimental tuberculosis in the guinea pig and to more fundamental changes produced in both the tubercle bacilli and the infected tissues.

It has been proved (1–4) that a therapeutic regimen consisting of isoniazid or streptomycin, started at the same time or a few days before inoculation, will not protect the experimental animals from dissemination of bacilli and production of tissue changes during the first stages of the disease. The present results indicate also that treatment with isoniazid and streptomycin cannot prevent such a dissemination, since the animals sacrificed 15 days after inoculation showed both gross and microscopic lesions in some organs, and these lesions were indistinguishable from the ones found in the control group. Tubercle bacilli were recovered from these organs, and the animals subinoculated with macerates of the same tissues developed progressive disease. Thus, experimental tuberculosis of two weeks' duration differs in almost no respect in treated and untreated animals; the only possible difference is the time of appearance of a positive tuberculin test. In the control untreated animals, the tuberculin reaction is positive 15 days after inoculation, whereas in the treated group the reaction is negative or doubtful at the same time and only becomes positive at 30 days.

It has been suggested that, to be effective, streptomycin requires the establishment of hypersensitivity (1). Furthermore, during the early phases of the disease many bacilli are intracellular, and it has been shown that streptomycin is not fully effective against them in that position (9). The present writers believe that in addition to these factors there may be others which might explain the ineffectiveness of the drugs in the early stages of experimental tuberculosis. It is possible that the drugs will require some time to reach adequate concentrations in the tissues and that hypersensitivity may change the reactivity of the tissues in such a way that drugs could act more efficiently.

In spite of the regression and disappearance of the lesions in guinea pigs treated 30 and 60 days, it is certain that the treatment did not eliminate all of the bacilli from *all* of the tissues. After the treatment was stopped, the lesions reappeared. This emergence of bacilli is peculiar, however, in that the microorganisms appeared first in the iliac lymph node, as if the animal had just been

inoculated. It was also interesting that in many cases the lesion was restricted to the lymph node for a long time, especially if the treatment lasted 60 days, since no lesions were found in other organs in the autopsy carried out 240 days after treatment was discontinued. This would suggest that the changes effected by the drugs on the bacilli are proportional to the duration of the treatment and that each organ shows a peculiar reaction to infection. The last suggestion could depend on the number of bacilli present in each organ at the time the treatment becomes effective. Tissues with fewer microorganisms would be easier for the drugs to sterilize, but such sterilization could also be due to a different defense mechanism, peculiar to each organ and not identical with the general mechanisms of immunity.

In the animals with prolonged treatment and further observation, the lesions were limited to the iliac nodes, and no bacilli were demonstrated by either smears or cultures. This phenomenon could be due to a real lack of dissemination of bacilli from the lymph node or, if dissemination occurred, the bacilli were rapidly eliminated by an increased ability of the tissues to destroy them.

Finally, a positive tuberculin test was obtained in animals subinoculated with suspensions of organs of guinea pigs inoculated and treated for 60 days, in which there were no gross or microscopic lesions and in which no bacilli were recovered by culture. Furthermore, when the tuberculin-positive animals were sacrified no lesions were detected in any organ and no bacilli were cultured from them. This poses an interesting question, since it seems established that a positive tuberculin test occurs only when there has been a tissue lesion. It appears, therefore, that there were lesions present but they were not detected because they were small and scarce; or that at the time of sacrificing the animals there were no demonstrable lesions because they were healed.

Summary

Guinea pigs infected with an appropriate dose of tubercle bacilli and treated since the day of infection with isoniazid and streptomycin for 15 and 30 days showed fewer lesions the longer the treatment was continued. A similar group of animals treated for 60 days failed to show any morphologic evidence of tuberculosis at the end of treatment. Two hundred and forty days after discontinuation of treatment, however, lesions appeared which were limited to the lymph nodes.

Different guinea pigs subinoculated with one of several organs of animals treated for 15 and 30 days developed tuberculosis. When subinoculation was carried out in the same manner with macerates of organs of animals treated for 60 days, no evidence of tuberculosis was present despite the fact that the subinoculated guinea pigs became tuberculin positive.

Sumario

Persistencia de los Bacilos Tuberculosos en los Organos de Cobayos bajo Quimioterapia

Los cobayos infectados con una dosis eficaz de bacilos tuberculosos y tratados desde el día de la infección con una combinación de estreptomicina e isoniacida durante quince y treinta días revelaron menos lesiones mientras más duraba el tratamiento. Un grupo semejante de animales tratados durante sesenta días no reveló ningún signo morfológico

de tuberculosis al terminar el tratamiento. No obstante, a los 240 días de discontinuarse el tratamiento, aparecieron lesiones que se limitaban a los ganglios linfáticos.

Diversos cobayos subinoculados con uno de varios órganos de los animales tratados durante quince o treinta días manifestaron tuberculosis. Cuando se llevó a cabo la subinoculación de la misma manera con preparaciones maceradas de órganos de los animales tratados durante sesenta días, no hubo signos de tuberculosis a pesar de que los cobayos subinoculados se habían vuelto positivos a la tuberculina.

Resume

Persistance des bacilles tuberculeux dans les organes du cobaye soumis à la chimiothérapie

Chez les cobayes inoculés avec une dose efficace de bacilles tuberculeux et traités dès le jour de l'inoculation par l'association streptomycine-isoniazide pendant 15 ou 30 jours, les lésions observées étaient moins nombreuses quand le traitement était le plus long. Un groupe similaire d'animaux traité pendant 60 jours ne présentait aucun signe morphologique de tuberculose à la fin du traitement, toutefois, 240 jours après l'arrêt du traitement, des lésions limitées aux ganglions lymphatiques sont apparues.

Un autre groupe de cobayes subinoculés avec des extraits d'organes d'animaux traités pendant 15 jours ou pendant 30 jours, la tuberculose s'est développée. Quand la subinoculation a été effectuée de la même manière avec des macérations d'organes d'animaux traités pendant 60 jours, aucun signe de tuberculose n'était présent en dépit du fait que la réaction à la tuberculine devenait positive chez les cobayes subinoculés.

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BIOLOGIC AND BIOCHEMICAL ACTIVITIES OF "CORD FACTOR"[1]

With Special Reference to Its Role in the Virulence of Tubercle Bacilli

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INTRODUCTION

In 1950, a lipid with toxic properties ("cord factor") was extracted from virulent tubercle bacilli by Bloch (1, 2). This compound has since been thoroughly purified (3, 4) and was identified by Noll and associates (5) as trehalose-6,6′-dimycolate. This structure was confirmed by synthesis (6, 7). Despite the extensive work on the chemistry of "cord factor," its biologic significance has remained more obscure. Based on a number of suggestive associations, indirect evidence indicates that "cord factor" played a part in the virulence of *M. tuberculosis* (2, 8–10). The suggestion, however, that "cord factor" acted as a growth-promoting agent during the early phase of infection (11) was not substantiated in these studies.

In an attempt to better understand the action of "cord factor," four types of experiments were designed to study the biologic and biochemical effects of "cord factor" and its possible role in virulence. They concerned the following aspects: the general toxicity of "cord factor" in albino mice; the effect of "cord factor" on the *in vivo* survival of avirulent human tubercle bacilli (strain H37Ra); the effect of "cord factor" on the *in vivo* survival and multiplication of virulent human tubercle bacilli (strain H37Rv) and its effect on the establishment of tuberculous lesions; and the effect of "cord factor" on some enzyme systems.

It was found that "cord factor" failed to influence the *in vivo* multiplication of tubercle bacilli, but it inhibited the succinic and malic dehydrogenase systems of mouse liver. These results are presented and discussed below.

MATERIALS AND METHODS

Animals: Inbred Swiss albino mice, strain Na-2, were used in all experiments. They were received in this laboratory at four weeks of age and maintained in separate cages in groups of 10 mice; they were fed pellet diet and water *ad libitum*. Male animals of various ages, according to the experimental design, were used.

Bacterial strains: Virulent human tubercle bacilli H37Rv and its avirulent variant H37Ra were used. Bacterial suspensions for mouse infection were prepared by the usual beads-flask technique with the surface growth on Sauton's synthetic liquid medium grown for two to three weeks. Large cell clumps in the suspension were removed by centrifugation at 1,500 r.p.m. for five minutes. The inoculum suspension contained chiefly single cells and a few small aggregates. The viable units in the inoculum were determined by plating serial dilutions of original suspension on glycerol-egg medium.

"Cord factor": The "cord factor" was a purified sample extracted from virulent human tubercle bacilli, strain Brévannes, obtained through the courtesy of Dr. H. Bloch. For

[1] From the Research Laboratory of Toneyama Hospital, National Sanatorium, Toyonaka, Osaka, Japan.

injection into mice, it was dissolved in olive oil and sterilized at 100°C. for thirty minutes. During the course of the experiments, it was maintained at 5°C. as 0.1 per cent solution in olive oil.

Determination of in vivo growth of bacilli: The homogenate technique of whole bodies of mice (12) was applied.

Determination of succinic dehydrogenase activity of mouse tissues: The colorimetric method developed by Kun and Aboad (13) was employed.

Results

The general toxicity of "cord factor": The toxicity of "cord factor" was tested by injecting mice intraperitoneally with 0.1 mg. dissolved in 0.1 ml. of olive oil once, or by injecting 0.01 mg. in 0.1 ml. of olive oil every fifth day, according to the method of Bloch (9).

The body weights of groups of 10 mice were determined every fifth day and the deaths were recorded. No histopathologic examinations of mouse organs were carried out, but gross changes visible at autopsy were recorded. The previous report of Bloch (1) called attention to hemorrhages of the lungs so that the lungs of dead mice were examined with special precautions. The results are summarized in tables 1 and 2.

All mice which received either 0.1 or 0.01 mg. per 0.1 ml. of olive oil showed a severe diarrhea and marked inactivity. They did not feed the day after injection. Injection of 0.1 mg. of "cord factor" killed one of 10 mice the day after injection, but the remaining animals of this group gradually recovered and gained body weight thereafter. The 0.01 mg. injections repeated every fifth day killed 8 of 10 mice until the thirty-fifth day. The body weight of this group did not increase. No deaths were observed in the control group which received the olive oil once or on every fifth day.

At necropsy, the most remarkable change was the peritoneal exudation and adhesion. In one mouse dead on the thirty-second day, a caseous-like substance was recognized at the injection site in the peritoneal cavity. The significance of this change is not clear. No hemorrhagic congestion of the lungs was visible in 9 dead mice.

The difference in susceptibility to the toxic action of "cord factor" between the

TABLE 1

Effect of "Cord Factor" on the Body Weight of Mice

Days After Injection	Olive Oil		"Cord Factor"	
	0.1 Ml. Once	0.1 Ml. Every Fifth Day	0.1 Mg. Once	0.01 Mg. Every Fifth Day
0	12.8 (*8*)	13.0 (*10*)	13.5 (*10*)	13.5 (*10*)
5	18.2 (*8*)	16.5 (*10*)	15.0 (*9*)	16.5 (*10*)
10	19.4 (*8*)	18.5 (*10*)	15.0 (*9*)	16.1 (*9*)
15	20.6 (*8*)	18.5 (*10*)	16.1 (*9*)	15.6 (*8*)
20	20.0 (*8*)	18.0 (*10*)	16.7 (*9*)	15.0 (*6*)
25	23.8 (*8*)	22.0 (*10*)	18.9 (*9*)	14.0 (*5*)
30	21.5 (*8*)	19.0 (*10*)	18.9 (*9*)	16.7 (*3*)
35	21.3 (*8*)	19.0 (*10*)	19.4 (*9*)	17.0 (*2*)

Figures represent the average body weight per mouse in each group. The numbers in parentheses indicate the number of mice surviving at the designated days after injection.

TABLE 2

DEATH OF MICE BY THE INTRAPERITONEAL INJECTION OF "CORD FACTOR" AND GROSS PATHOLOGIC CHANGES OF THE DEAD MICE

Number of Deaths Until 35th Day	"Cord Factor" 0.1 Mg. Once 1/10	"Cord Factor" 0.01 Mg. Every Fifth Day 8/10
Days after injection		
1	1 died; markedly emaciated and weakened with severe diarrhea, exudate in peritoneal cavity; no change in lungs.	
10		2 died; peritoneum and peritoneal organs adhered tightly; intestinal cord became a conglomerate; no change in lungs.
11		
16		2 died. Not examined at necropsy.
21		1 died; peritoneum very thick; adhesion very marked; no change in lungs.
29		2 died. Not examined at necropsy.
32		1 died; peritoneum thick; portal and mesenterial lymph nodes swollen in yellowish-white masses. Under the injection site a bean-size tumor containing yellowish-white caseous substance was recognized. It was amorphous and nonacid fast, containing a few leukocytes with inspissated nuclei but no bacteria. Spleen was enlarged; liver and stomach adhered tightly; no change in lungs.

"Cord factor" was dissolved in 0.1 ml. of olive oil and injected into mice intraperitoneally. Olive oil caused no gross changes even by repeated injections.

strains of mice employed must be considered since, in Bloch's experiments (1), hemorrhages were most conspicuous with the C57 strain. As shown below, the CF1 strain used by Bloch and the Na-2 strain were equally susceptible to the injection of "cord factor." A precise histopathologic examination would be necessary on this question of pulmonary hemorrhage.

The effect of "cord factor" on the in vivo survival of avirulent bacilli H37Ra: As shown in table 3, the injection of "cord factor" did not influence the fate of H37Ra in the whole bodies of mice. The difference of the death rates between the next two experiments and the one reported in figure 1 is due to the weekly sacrifice of 2 mice before the occurrence of death caused by the injection of "cord factor."

The effect of "cord factor" on the in vivo multiplication of virulent human tubercle bacilli H37Rv and its effect on the establishment of tuberculous lesions: Duplicate

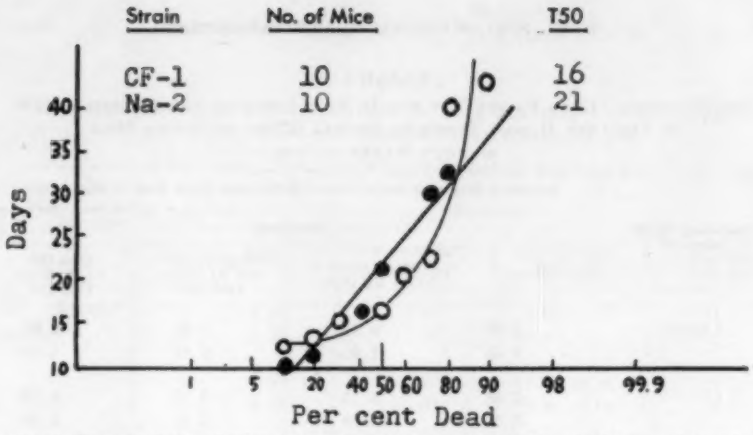

FIG. 1. Rate of death of mice by the intraperitoneal injection of 0.01 mg. of "cord factor" repeated every fifth day. The cumulative rate of death produced by the injection of "cord factor" was plotted on arithmetic-probability scale. The figures for CF1 mice were cited from the work of Bloch (9) for the sake of comparison.

TABLE 3

THE EFFECT OF "CORD FACTOR" ON THE IN VIVO SURVIVAL OF AVIRULENT HUMAN TUBERCLE BACILLI H37Ra

Weeks After H37Ra Infection*	Number of Recovered Viable Units of H37Ra from Whole Body of Mice ($\times 10^6$)			
	Treatment†			
	None	"Cord Factor" 0.1 Mg. Once	"Cord Factor" 0.01 Mg. Every Fifth Day	Olive Oil 0.1 Ml. Every Fifth Day
3 hours	7.64	4.32	7.19	5.42
	6.03	6.78	7.68	6.00
1	8.74	8.40	8.32	6.27
	7.76	8.84	5.70	5.59
2	7.10	11.30	7.95	4.45
	5.76	13.00	7.33	5.08
3	1.23	1.80	3.10	2.78
	1.36	2.85	1.05	1.98
4	0.57	2.10	1.41	0.27
	1.28	1.58	2.72	0.56
6	0.24	0.75	—	0.15
	0.38	—	—	0.44

* The mice weighing 18.0 (16.2–19.6) gm., six weeks of age, were infected intravenously with 4.2×10^6 viable units of H37Ra.

† 0.1 and 0.01 mg. of "cord factor" were injected intraperitoneally, dissolved in 0.1 ml. of olive oil.

— Dead during the course of the experiment.

TABLE 4

THE EFFECT OF "CORD FACTOR" ON THE IN VIVO SURVIVAL AND MULTIPLICATION OF VIRULENT HUMAN TUBERCLE BACILLI H37Rv IN YOUNG MICE OF FOUR WEEKS OF AGE

Weeks After H37Rv Infection*	Number of Recovered Viable Units of H37Rv from Whole Body of Mice ($\times 10^5$)			
	Treatment†			
	None	"Cord Factor" 0.1 Mg. Once	"Cord Factor" 0.01 Mg. Every Fifth Day	Olive Oil 0.1 Ml. Every Fifth Day
3 hours	3.96	0.84	3.00	3.63
	4.49	1.40	2.13	4.48
1	3.55	5.15	5.12	8.72
	5.54	9.78	3.17	5.66
2	128.00	21.00	26.40	39.40
	62.00	141.00	30.40	58.00
3	85.30	—	117.00	71.50
	107.00	—	83.80	85.50
4	3,260.00	—	153.00	726.00
	455.00	—	296.00	1,150.00
6	990.00	—	448.00	57.00
	4,430.00	—	2,620.00	292.00

* 15 mice in each group weighing 10.8 (10.0–11.5) gm., four weeks of age, were infected intravenously with 1.0×10^5 viable units of H37Rv.

† 0.1 mg. of "cord factor" was injected intraperitoneally into mice, dissolved in 0.1 ml. of olive oil, the day before the virulent infection. This injection alone killed 9 of 15 mice.

— Dead during the course of experiment.

tests on the effect of "cord factor" upon virulent infection of mice were done. First, mice at four weeks of age weighing 10 gm. were infected with 1.0×10^5 viable units of H37Rv, and the effect of "cord factor" was examined. Second, older mice at eight weeks of age weighing 18 gm. were infected with 9.8×10^6 viable units of the same strain.

In parallel with the determination of the total *in vivo* population size after virulent infection, the organ involvement of the control and "cord factor"-treated animals was compared macroscopically.

As evident from the results illustrated in table 4, the injection of "cord factor" did not accelerate the *in vivo* growth of H37Rv.

In accordance with these results, the concurrent injection of "cord factor" with virulent bacilli caused no aggravation of tuberculous lesions visible in lungs, spleen, liver, and lymph nodes.

The 0.1 mg. injection of "cord factor" alone, injected the day before virulent infection, killed 9 of 15 mice. As generally accepted, young mice are more susceptible to tuberculous infection than older ones.

TABLE 5

THE EFFECT OF "CORD FACTOR" ON THE IN VIVO SURVIVAL AND MULTIPLICATION OF H37Rv IN ADULT MICE OF EIGHT WEEKS OF AGE

Weeks After H37Rv Infection*	Number of Recovered Viable Units of H37Rv from Whole Body of Mice ($\times 10^5$)			
	Treatment†			
	None	"Cord Factor" 0.1 Mg. Once	"Cord Factor" 0.01 Mg. Every Fifth Day	Olive Oil 0.1 Ml. Every Fifth Day
3 hours	2.58	9.50	3.82	11.1
	3.55	12.10	2.15	12.1
1	6.65	34.40	11.8 (5.05)‡	12.5
	9.75	12.40	8.56 (6.93)	11.9
2	79.00	79.80	81.50	43.8
	81.60	42.50	81.00	61.6
3	55.40	98.50	160.00	46.2
	69.10	100.00	—	60.4
4	210.00	34.90		61.5
	100.00	—		14.0

* 10 mice weighing 18.0 (17.5–18.1) gm., eight weeks of age, in each group were infected intravenously with 9.8×10^6 viable units of H37Rv.

† 0.1 and 0.01 mg. of "cord factor" was injected intraperitoneally, dissolved in 0.1 ml. of olive oil.

‡ Figures in parentheses represent the viable units in whole bodies of mice immediately after death.

Comment: The high death rate in young mice by the one single injection of 0.1 mg. of "cord factor" is very interesting in this connection. This seems to mean that the injection of "cord factor" primarily inhibits some functions of hosts which are essential for survival of mice. Furthermore, the accumulation of toxic substances such as "cord factor" may play, whether specifically or not, an important role in the death of mice from tuberculous infection. This assumption leads to the enzymologic experiments described in the next section.

The effect of "cord factor" on the virulent infection of mice weighing 18 gm. at eight weeks of age is shown in table 5. The results are essentially the same as those presented in table 4. In this case, each group contained 10 mice. The one single injection of 0.1 mg. of "cord factor" did not kill mice, but 2 mice died in the 0.01 mg. group one week after the injection, and the total number of bacilli was estimated immediately after death. It is interesting that the the number of living bacilli in the whole body of mice immediately after death was not necessarily far greater than that in the surviving mice.

No macroscopic differences were seen between the lungs of mice treated with "cord factor" and the lungs of untreated animals. Tubercles developed three weeks after virulent infection and the spleens were enlarged two to three weeks later to about the same extent in both groups of animals.

The effect of "cord factor" on the succinic and malic dehydrogenase systems of

mouse tissues: From the acute toxic manifestation of "cord factor" (death of young mice after one single intraperitoneal injection of 0.1 mg. of this substance, marked loss of body weight, and inactivity of mice after the same injection), the following experiments were designed to examine the primary toxicity of "cord factor."

The succinic and malic dehydrogenase activities of kidneys and livers of mice which received 0.1 mg. of "cord factor" by the intraperitoneal route and showed marked inactivity about twenty-four hours later were determined by the tetrazolium technique of Kun and Aboad (13). Experimental groups were killed, together with the control animals which received 0.1 ml. of olive oil intraperitoneally, twenty-four hours after the injection of 0.1 mg. of "cord factor" in 0.1 ml. of olive oil. Ten per cent tissue homogenate was prepared in the presence of 0.1 M phosphate buffer (pH, 7.4).

System: phosphate buffer (0.1 M, pH, 7.4)	0.5 ml.
0.2 M succinate or 0.4 M DL-malate	0.5 ml.
10 per cent tissue homogenate	1.0 ml.
0.1 per cent triphenyltetrazolium chloride	1.0 ml.

The optical density of the reduced formazan was determined at 480 μ using Hitachi spectrophotometer after ten to thirty minutes' incubation at 37°C. and the extraction of the dye with acetone. From the standard curve, the γ of reduced formazan per 10 mg. of tissue per ten minutes was estimated.

The results are summarized in tables 6, 7, and 8.

It is clear from these results that "cord factor" decreases the activity of the succinic and malic dehydrogenase systems in the livers of mice about twenty-four hours after the intraperitoneal injection. The specificity of this action is to be investigated in the further studies. The mechanism of this decrease is not clear with the available data at present, but the scheme designated below of the hydrogen transfer systems from succinate and malate seems to be suggestive.

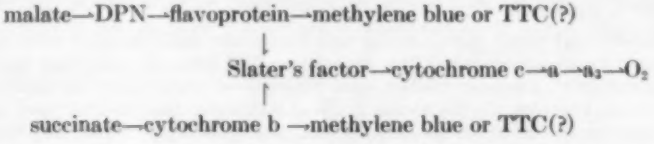
malate—DPN—flavoprotein—methylene blue or TTC(?)
Slater's factor—cytochrome c—a—a3—O2
succinate—cytochrome b —methylene blue or TTC(?)

TABLE 6

SUCCINIC DEHYDROGENASE ACTIVITIES OF LIVER AND KIDNEYS OF MICE AFTER THE INTRAPERITONEAL INJECTION OF "CORD FACTOR"

Treatment*	Mean Loss of Body Weight	γ of Formazan Reduced per 10 Mg. of Tissue per 10 Minutes	
	gm.		
Olive oil	+0.7	Kidneys	4.2 ± 1.20
		Liver	5.3 ± 0.07
"Cord factor"	−2.9	Kidneys	5.2 ± 0.3
		Liver	3.3 ± 0.4†

Both groups of mice consisted of 5 animals.

* 0.1 ml. of olive oil or 0.1 mg. of "cord factor" dissolved in 0.1 ml. of olive oil was injected intraperitoneally 25 hours before the estimation of enzyme activity.

† $\alpha = 0.01$

TABLE 7

DECREASE OF SUCCINIC DEHYDROGENASE ACTIVITY OF LIVER OF MICE AFTER THE INTRAPERITONEAL INJECTION OF "CORD FACTOR"

Number of Mice	γ of Formazan Reduced per 10 Mg. of Tissue per 10 Minutes			
	Treatment*			
	Olive Oil		"Cord Factor"	
	Kidneys	Liver	Kidneys	Liver
1	6.2	11.2	5.6	6.8
2	6.3	9.3	5.8	3.7
3	6.2	13.8	5.9	6.0
4	5.8	7.8	—	6.1
5	5.5	9.2	5.6	5.7
6		11.4		6.1
7		8.5		5.8
8		8.3		5.9
9		10.2		7.9
10		12.5		5.5
Mean	6.0 ± 0.1	9.75 ± 2.7	5.7 ± 0.2	6.1 ± 1.2†

* 0.1 ml. of olive oil or 0.1 mg. of "cord factor" dissolved in 0.1 ml. of olive oil was injected intraperitoneally 22 hours before the estimation of enzyme activity.

† $\alpha = 0.01$

TABLE 8

DECREASE OF ACTIVITY OF SUCCINIC AND MALIC DEHYDROGENASE SYSTEMS IN LIVER OF MICE AFTER THE INTRAPERITONEAL INJECTION OF "CORD FACTOR"

Number of Mice	γ of Formazan Reduced per 10 Mg. of Liver per 10 Minutes			
	Treatment*			
	Olive Oil		"Cord Factor"	
	Succinate	Malate	Succinate	Malate
1	3.2	1.0	1.3	0.8
2	2.6	1.9	0.8	1.0
3	3.4	1.3	1.3	1.0
4	3.5	2.3	1.0	0.5
5	4.8	3.0	2.5	0.5
Mean	3.7 ± 0.25	1.9 ± 0.3	1.4 ± 0.3†	0.76 ± 0.1†

* 0.1 ml. of olive oil or 0.1 mg of "cord factor" was injected intraperitoneally 26 hours before the estimation of enzyme activity.

† $\alpha = 0.01$

An investigation is now in progress on the mechanism of this decrease of activity in the hydrogen transferring system.

Martin and associates (14) have reported a decrease of succinic dehydrogenase activity in the kidneys of tuberculous guinea pigs. It is interesting to investigate the correlation between the course of tuberculous infection of mice and the de-

crease of the activity of liver enzyme systems. It may be assumed that the "cord factor" plays the important role in the decreasing of these enzyme activities and is responsible for the inactivation and death from infection. A more precise series of studies on this problem is now in progress.

Summary

Using the homogenate technique of whole body of mice, it was shown that "cord factor" did not influence the survival and multiplication of either virulent or avirulent human tubercle bacilli in mouse bodies. The toxicity of "cord factor" for mice was confirmed.

"Cord factor" caused a significant decrease in the activity of the succinic and malic dehydrogenase systems of the liver of mice about twenty-four hours after the intraperitoneal injection of 0.1 mg.

Sumario

Actividades Biológicas y Bioquímicas del "Factor Cordón," con Referencia Particular a su Papel en la Virulencia de los Bacilos Tuberculosos

Usando la técnica homogeneada de cuerpos enteros de ratones, se demostró que el "factor cordón" no afectaba la sobrevivencia y multiplicación de los bacilos tuberculosos humanos ya virulentos o avirulentos en los cuerpos de ratones. Se confirmó la toxicidad del "factor cordón" para los ratones.

El "factor cordón" ocasionó una disminución importante en la actividad de los sistemas succínico y mélico de dehidrogenasa del hígado de los ratones unas veinticuatro horas después de la inyección intraperitoneal de 0.1 mg.

Resume

Activités biologiques et biochimiques du "Cord factor" et plus spécialement son rôle dans la virulence du bacille tuberculeux

Au moyen de l'homogénéisation du corps entier de la souris, les auteurs ont démontré que le "cord factor" n'influençait ni la survie, ni la reproduction des bacilles tuberculeux virulents ou avirulents dans l'organisme de la souris. La toxicité du "cord factor" pour la souris a été confirmée.

Le "cord factor" causait une réduction notable de l'activité des systèmes enzymatiques malico-déhydrase et succino-déhydrase du foie de la souris, environ vingt-quatre heures après l'injection intrapéritonéale de 0,1 mg.

Acknowledgment

The writers wish to express their gratitude to Dr. H. Bloch for supplying the purified sample of "cord factor" for this study.

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Case Reports

ACUTE ANAPHYLACTIC SHOCK DUE TO PARA-AMINOSALICYLIC ACID[1]

H. P. B. HARVEY AND H. J. SOLOMON

(Received for publication July 9, 1957)

INTRODUCTION

In the treatment of tuberculosis the need is constantly recurring to suspend or modify chemotherapy when undesirable side effects develop to the drugs in use.

The possibility of a fulminating anaphylactic reaction to para-aminosalicylic acid (PAS) is not well recognized and such an episode seems worth reporting.

CASE REPORT

The patient, a man forty-eight years old, was admitted to the hospital on July 15, 1956, because of a sudden hemoptysis (500 ml.).

He was known to have had pulmonary tuberculosis since 1949 and during the preceding seven years had spent most of his time in various sanatoriums. In 1949 the patient had commenced treatment with 12 gm. of sodium PAS daily and 1 gm. of streptomycin intramuscularly twice weekly. He had remained on these drugs for three years, during the last year of which 300 mg. of isoniazid per day had been added.

The patient stated that on two occasions within an hour of his streptomycin injections he had felt weak, had perspired freely, and had had a "blackout." He evidently recovered quickly on each occasion, and therapy was continued until 1952.

In 1954 further chemotherapy had been started but had been immediately stopped when another "blackout" followed the first dose of 4 gm. of PAS. No record is available whether streptomycin was also given, but the patient thinks not and feels sure that PAS caused this quite severe episode. No further PAS was given until after the present admission.

In June, 1956, it was decided in another clinic to administer further chemotherapy; 1 gm. of streptomycin was given intramuscularly and PAS was prescribed. Apparently, none of the latter drug had been taken when the patient was brought back into the clinic one hour later. The only available report states: "The patient was in a collapsed state, cyanosed, and pulseless. He responded to the administration of oxygen, brandy, and intramuscular Anthisan®." Antimicrobials were then suspended.

During the twenty-four hours after admission for this latest hemoptysis, he suffered further bleeding amounting to an additional 500 ml. of blood, after which hemorrhage ceased.

The patient was not in shock and settled down well. Three days later his hemoglobin was 13.1 gm. per 100 ml. and his blood pressure was 135 systolic, 95 diastolic in mm. of mercury. A roentgenogram of his chest showed no change from one taken two months previously, and no acid-fast bacilli were demonstrated in his sputum.

It was decided to define his drug hypersensitivity and possibly to attempt desensitization. As the history indicated that the chief reactions had been to streptomycin, it was decided first to check his reaction to PAS. A trial dose to 0.5 gm. of sodium PAS orally was ordered and, by a misunderstanding, 1.5 gm. were given. The consequences were dramatic. Fifteen minutes later the patient complained of severe cramping pain in the abdomen which radiated up into the chest. He had two profuse, watery bowel movements, commenced to vomit, and looked apprehensive and shocked. His blood pressure was 40 systolic, 20 diastolic and the apex rate was 120 per minute. His skin was cold and cyanosed

[1] From the Thoracic Unit, Royal Prince Alfred Hospital, Sydney, Australia.

and he was sweating profusely. The foot of the bed was raised and 30 mg. of desoxyephedrine were administered intravenously. This only succeeded in raising the blood pressure to the level of 80 systolic, 50 diastolic for a period of five minutes, after which it fell to zero and his condition appeared critical. An intravenous infusion of 0.8 mg. of norepinephrine per 100 ml. of 5 per cent glucose was commenced at a rate of 40 drops per minute. It was quickly found that this was insufficient, and 60 drops per minute of a 2.4 mg. per 100 ml. solution were required to maintain the blood pressure near 120 systolic, 80 diastolic. At times the systolic pressure would rise to 200 mm. and the patient would complain of severe headache, while at other times it would fall to 80, at which low level the patient complained of cramps in the calves of the legs. After two hours the norepinephrine requirement dropped, the patient's general condition improved, symptoms abated, and the blood pressure became more stable.

At this stage an absolute eosinophilic count on peripheral blood showed 69 eosinophils per cu. mm. In the two hours during which the patient was critically ill, the chances of recovery seemed remote.

For the next twenty hours the blood pressure was easily maintained by 20 drops per minute of an 0.4 mg. per 100 ml. solution of norepinephrine, but fell steeply if this was withdrawn. Thereafter it was found that the patient felt quite well and could again maintain normal blood pressure levels unaided.

He has suffered no sequelae from this episode other than a permanent mistrust of all antituberculous drugs.

Discussion

Reactions to PAS are unfortunately very frequent. Gastrointestinal intolerance is by far the most common and is experienced in some degree by most patients on doses of more than 12 gm. per day.

This represents a simple toxic phenomenon since its frequency and severity vary directly with the size of the dose administered. Other toxic manifestations related to dosage are a mild hypoprothrombinemia similar to that produced by any of the salicylates (1), and a mild disturbance of thyroid function which may have a goitrogenic effect (2).

In a proportion of cases which Hayes and Weiss (3) report to be about 4 per cent of any large series, side reactions occur which seem to be on a hypersensitivity basis. They are often brought on by a very small dose and have on some occasions responded to desensitization. Drug fever and skin rashes are by far the commonest of these and nearly always accompany the other variations (4).

Many other reactions believed to be due to hypersensitivity to PAS have been reported either alone or in various combinations. Warring and Howlett (5), Hayes and Weiss (3), and Paine (6) in recent reviews include in their lists: headache and meningismus; syndrome of burning eyes, lacrimation, and conjunctivitis; Löffler's syndrome; angioneurotic edema; spasmodic cough and wheezing; granulopenia; eosinophilia; lymphadenopathy; an infectious mononucleosis syndrome with a negative Paul-Bunnell test; exfoliative dermatitis; and hepatitis.

These latter two types have accounted for all of the fatal PAS reactions so far reported. In these cases death is delayed and occurs in five (4) to eighteen days (7).

Extremely rarely a typical acute anaphylactic reaction occurs such as occurred in this patient, although no reports of immediate death could be found in the literature. One patient reported by Bulley (8) developed acute circulatory failure combined with limb cramps, severe back pain, headache, vomiting, and diarrhea. She narrowly survived to develop the Guillain-Barré syndrome.

The patient in the case reported by Bulley had previously suffered headache, nausea, shivering, malaise, and fever to 102.4° F. after each of a number of previous doses of PAS, and, in the nearly fatal anaphylactic reaction reported, developed symptoms within ten

minutes of an oral dose of 4 gm. Apart from the late development of the Guillain-Barré syndrome, the course of the episode was almost identical with that in the present case. Fortunately each patient survived although not without the use of vigorous resuscitative measures. In the present case recovery could not have been expected without the use of norepinephrine and, even so, many anxious moments were experienced.

Undoubtedly far too large a test dose was used in the case reported here, partly due to a dosage error, but chiefly due to failure to appreciate the significance of previous episodes of a "shock" state following PAS ingestion and to realize the occasional lethal potentiality of this drug. Hayes and Weiss recommend a 50 mg. test dose of PAS when hypersensitivity is suspected and, although there is no guarantee that even this is safe, such a dose is now being given in this hospital.

The closely related substances sodium salicylate and acetylsalicylic acid have a well-recognized propensity to provoke acute anaphylaxis, and a number of sudden deaths following the ingestion of "aspirin" have been recorded (9). Presumably the mechanism of the production of PAS anaphylaxis is the same and it would be interesting to know whether there is any cross-hypersensitivity. No mention is made of the effects of other salicylates on Bulley's patient, although a 29-year-old housewife is likely to have some contact with the drugs.

In the present case the patient was quite sure that he had not taken "an aspirin" or "headache powder" for many years and few, if any, during his life. No attempt to test for sensitivity to these drugs was made after the alarming episode described, but he was strongly advised to avoid forever all forms of acetylsalicylic acid and allied drugs.

Summary

A man with pulmonary tuberculosis developed profound shock, diarrhea, abdominal pain, headache, and muscular cramps within twenty minutes of ingestion of a test dose of 1.5 gm. of PAS. Recovery followed twenty-four hours of continuous administration of intravenous fluids and norepinephrine.

A large number of sensitivity reactions to PAS have been reported, but only one other case of severe anaphylactic shock similar to that which occurred in the patient of the present case could be found in the literature.

Prior to this reported reaction, hypersensitivity to both streptomycin and PAS seemed very likely. It is recommended that in these circumstances a small test dose of PAS no larger than 50 mg. be employed in future.

The close similarity of PAS to the acetylsalicylic acid group of drugs which are well known to cause occasional acute anaphylaxis makes it reasonable to assume that cross-hypersensitivity might well exist and patients with hypersensitivity to one of the salicylates should be warned of the dangers of the other members of the group.

Sumario

Choque Anafiláctico Agudo Debido al Acido Para-Aminosalicílico

Un sujeto con tuberculosis pulmonar manifestó choque profundo, diarrea, dolor abdominal, cefalalgia y calambres musculares en término de veinte minutos de la ingestión de una dosis experimental de 1.5 gm. de PAS. La reposición vino tras veinticuatro horas de continua introducción endovenosa de líquidos y noradrenalina.

Se ha descrito un gran número de reacciones de hipersensibilidad al PAS, pero en la literatura no pudo encontrarse más que otro caso de choque anafiláctico grave, semejante al observado en el enfermo del caso actual.

Antes de esta reacción, parecía muy probable que existiera, según se había mencionado,

susceptibilidad tanto a la estreptomicina como al PAS. Recomiéndase que, en tales circunstancias, no se empleee en el futuro más que una pequeña dosis experimental de PAS, que no exceda de 50 mg.

Resume

Choc anaphylactique intense dû à l'acide para-aminosalicylique

Un homme atteint de tuberculose pulmonaire a présenté un état de choc intense, avec diarrhée, douleurs abdominales et crampes musculaires vingt minutes après avoir absorbé une dose d'essai de 1.5 g de PAS. La guérison a été obtenue au bout de vingt-quatre heures de traitement par l'injection intraveineuse continue de liquides et de noradrénaline.

Un grand nombre de réactions allergiques au PAS ont été rapportées, mais, en dehors du cas mentionné ci-dessus, il n'y a qu'un seul cas de choc anaphylactique aussi grave qui ait été publié.

Une hypersensibilité à la streptomycine et au PAS antérieure à cette réaction paraît avoir existé. Dans ces circonstances, on conseille désormais d'administrer une dose d'essai de PAS ne dépassant pas 50 mg.

Acknowledgment

The writers wish to thank Dr. Cotter Harvey, under whose care this patient was admitted.

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HEMANGIOPERICYTOMA OF THE LUNG[1]

SEYMOUR OCHSNER AND PAUL T. DECAMP

(Received for publication September 30, 1957)

INTRODUCTION

In 1942, Stout and Murray (1) published the original description of the mesenchymal tumor that they named hemangiopericytoma. They believed that these tumors resulted from abnormal proliferation of a type of cell (pericyte) normally in the outer sheath of capillaries and that they should be segregated into a separate category. In 1949, Stout (2) reported twenty-five additional cases that he had encountered since his original report of eight cases. As these tumors arise from capillaries, they may occur in any part of the body, although most of the reported cases were discovered in the superficial soft parts. Stout stressed the malignant potential of these tumors.

Additional reports have featured hemangiopericytomas in various locations, including the retroperitoneal space (3), sigmoid colon (4), rectum (5), and meninges (6). As far as the present writers have been able to determine, the case to be presented is the second that has been reported to have occurred primarily within the lungs. In the first case (7), the tumor, which caused repeated hemoptysis, was excised successfully, and the patient has remained in good health.

CASE REPORT

R. M., a Negro railroad laborer, 38 years old, was seen at the Ochsner Clinic on June 4, 1951, because of recurrent hemoptysis of thirteen years' duration. During the preceding three months the episodes had become more frequent, and copious amounts of blood were expectorated. A chronic cough had developed, and on several occasions the patient had coughed up what seemed to be pieces of "meat." Two months before admission the patient had been sent to a sanatorium, where an endobronchial lesion had been seen in the right bronchus on bronchoscopic examination. No tubercle bacilli were found in the examined sputum. Two years before admission he had been treated with penicillin for a syphilitic infection.

On physical examination on admission, some inconstant rhonchi were detected. An ill-defined density in the right lower lobe involving the subhilar portion of the lung was seen in the roentgenogram of the chest made on June 4, 1951 (figure 1). The hematocrit was 33 per cent and hemoglobin 8.5 per 100 ml. The sedimentation rate was 70 mm. in one hour by the Westergren method.

The patient was admitted to the Flint Goodridge Hospital in New Orleans, where a right pneumonectomy was done on June 11, 1951. A tumor measuring 8 by 6 cm. was seen in the lower lobe of the right lung with an extension protruding into the bronchial tree at the orifices of the medial basal and posterior basal segmental bronchi. The pathologist reported the tumor to be a hemangiopericytoma of the lung (figure 2).

Postoperative recuperation was rapid, and the patient was discharged on the tenth day after pneumonectomy. In February, 1952, he had several episodes of blood-streaked sputum. A small zone of granulation at the bronchial suture line was discovered on bronchoscopic examination. This was cauterized, and the patient has had no further hemoptysis. On March 14, 1953, he was well and working, and no abnormalities were seen in the roentgenogram of the chest. In June, 1957, information was obtained that he has remained in satisfactory health.

[1] From the departments of Radiology and Surgery, Ochsner Clinic, New Orleans, Louisiana.

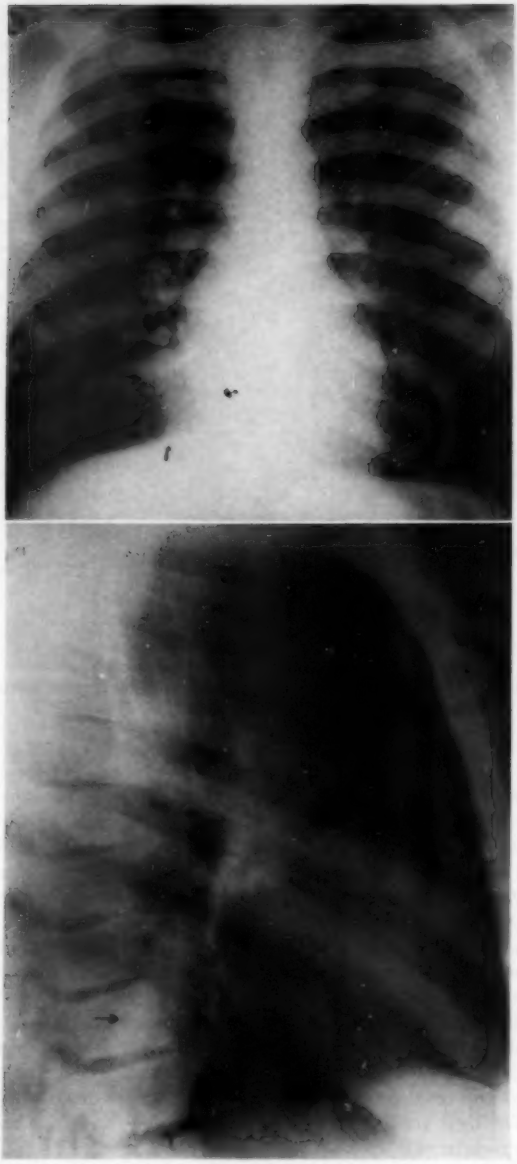

FIG. 1. Roentgenograms of thorax. (Upper) Frontal view shows mass below right hilum apparently in cardiophrenic angle. (Lower) Lateral view indicates that the lesion is in the right lower lobe.

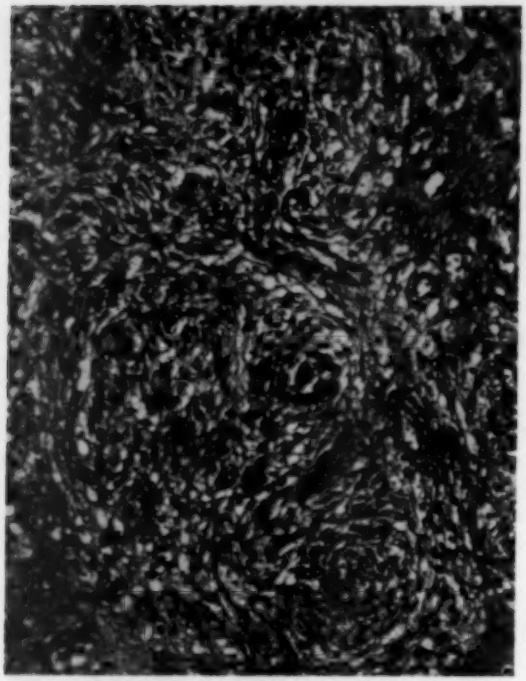

FIG. 2. Photomicrograph shows cellular type and architecture of hemangiopericytoma in the lung. (× 100)

COMMENT

Pathologic considerations: Stout and Murray (1) considered hemangiopericytomas to be vascular tumors featured by proliferation of capillaries, surrounding which were massed spindle-shaped or round cells somewhat in the fashion of the vessels and cells of glomus tumors. Unlike glomus tumors, they do not have the highly organoid architecture, the encapsulation, the remarkable number of nerves, or the associated paroxysms of pain. The characteristic cell is the pericyte, originally described by the Swiss histologist Zimmermann (8) as a modified smooth-muscle cell. It is contractile and closely applied to the capillary wall, and has an assumed function of altering the size of the lumen of the capillary.

Stout (2) described the features of the complex histologic appearance, perivascular orientation of the pericyte, profuse proliferation of obvious or occult capillaries, and the assistance in diagnosis afforded by a reticulum stain that would blacken the capillary sheaths and make recognition easier. Grossly, the tumors are described as often having a distinct tan color, their vascularity is usually not notable, and they are generally firm and irregular or nodular (4).

The incidence of malignant growth is important. In six of Stout's (2) thirty-three cases there was definite proof of malignant degeneration, and one patient had pulmonary metastasis. In five of the fourteen cases reported by McCormack and Gallivan (7), ulti-

mate pulmonary metastasis occurred. In two of these cases an apparently solitary metastatic extension was successfully resected.

The occurrence of hemangiopericytomas in widespread locations is not surprising in view of the capillary origin of the tumor. Hemangiopericytomas have been reported in all age groups (2, 7, 9). The presenting complaint is usually the presence of a visible or palpable mass. The lesion is generally painless unless it causes pressure on adjacent tissues or nerves. Its size may remain apparently stationary for long periods (sixty years in the case of Stout and Cassel (9) of an omental hemangiopericytoma), may grow slowly, or may metastasize widely. Although of vascular origin, these tumors rarely cause bleeding. The patient of the present case did suffer recurring attacks of hemoptysis. Calcifications may be seen in the tumor when it is visualized roentgenographically (3, 10).

Prompt excision is the only known logical method of therapy (11). Despite the occasional long history of a painless or apparently stationary mass, the tumor has such a definite malignant potential that it should be resected as soon as discovered. Kent (10) stressed the value of roentgenotherapy in nonresectable, incompletely excised, recurrent, or metastatic hemangiopericytomas.

Summary

A case of hemangiopericytoma of the lung has been recorded, apparently the second to be reported in the literature. The tumor caused recurrent hemoptysis for many years, was visualized by roentgenographic study as a pulmonary mass below the right hilum, was seen on bronchoscopic study, and was successfully removed by pneumonectomy. The patient has remained well for more than six years.

Sumario

Hemangiopericitoma del Pulmón

Se describe un caso de hemangiopericitoma del pulmón, aparentemente el segundo que consta en la literatura. El tumor provocó hemoptisis recurrente por muchos años, fué visualizado como tumefacción pulmonar debajo del hilio derecho con el estudio roentgenográfico, fué observado al hacer el estudio broncoscópico y fué extirpado sin complicaciones con la neumonectomía. El enfermo ha permanecido bien durante más de seis años.

Resume

Hémangiopéricytoma du poumon

Un cas d'hémangio-péricytoma du poumon est enregistré; il semble que ce soit le second qui ait été publié. La tumeur, qui avait provoqué l'hémoptysie à répétition durant de nombreuses années, a été révélée par l'exploration radiologique sous la forme d'une masse pulmonaire située au-dessous du hile droit; celle-ci a été observée par bronchoscopie et réséquée par pneumonectomie. L'état du malade est resté bon pendant plus de six ans.

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Notes

STUDIES ON THE MODE OF ACTION OF ISONIAZID

I. The Role of Trace Metals in the Inhibition of Bovine Liver Catalase by Isoniazid

In an earlier communication[1] the literature pertinent to the mode of action of isoniazid was briefly reviewed. Based on this review plus preliminary data from a pilot type of study, a theory was advanced to explain the mode of action of the antimicrobial action of isoniazid on *M. tuberculosis*. The theory simply states that the mode of action involves first the chelation of isoniazid with a trace metal. The structure of the resulting chelate enables it to compete successfully with hydrogen peroxide for the substrate sites on the catalase molecule. This would result in an accumulation of hydrogen peroxide which is highly toxic for the bacilli. An extensive investigation of this theory was planned and is currently in execution in this laboratory. The burden of the present paper is to describe investigations of the kinetics of bovine liver catalase, its inhibition by isoniazid, and the role therein of certain trace metals.

Since the publication of the earlier communication,[1] an article by Garattini and Leonardi[2] has come to the attention of the present writers. This paper describes their investigation of the antituberculous activity and toxicity of a series of chelating agents. Two of these compounds, 1,10-phenanthroline and diethyldithio-carbamate, showed antituberculous activity. The latter is less toxic than isoniazid.

The catalase employed in the present study was technical grade, bovine liver catalase.[3] The concentration was adjusted so that not more than one-fifth of the substrate (H_2O_2) originally present in the inhibitor-free reference was decomposed in one minute at 4°C. All observations were made using 10 ml. of 0.1 M phosphate buffer, pH 7.0, and 10 ml. of 0.1 M hydrogen peroxide.

A titration of unused hydrogen peroxide was employed in the determination of the catalase activity shown in all of the figures. In this procedure 0.5 ml. of the catalase solutions was added to the hydrogen peroxide in the phosphate buffer. One minute later the reaction was stopped by the addition of 5 ml. of 20 per cent sulfuric acid. The unused hydrogen peroxide was then determined titrimetrically with 0.05N potassium permanganate. The results are expressed as per cent of the activity of the inhibitor-free enzyme.

In all of the experiments involving an inhibitor, an incubation period of at least one hour was employed, during which the inhibitor was in contact with the enzyme before the addition of substrate.

The results of a study of the effects of 12 metal ions on the inhibition of bovine liver catalase by isoniazid are presented in figure 1. The results shown were confirmed on two occasions. It should be noted that the only ions which did not appreciably increase the inhibition were silver and cuprous ion, the only monovalent ions in the series. Moreover, chromium ion was the only metal ion in the series which did not inhibit catalase independently of isoniazid. The concentration of both isoniazid and the metal ions in this experiment was 5×10^{-3} M. The stippled control contained isoniazid alone (no metal).

[1] Maher, J. R., *et al.*: Am. Rev. Tuberc., 1957, *75*, 517.

[2] Garattini, S., and Leonardi, A.: Giorn. ital. chemoterap., 1955, *2*, 18.

[3] A product of Nutritional Biochemicals Corp., Cleveland, Ohio.

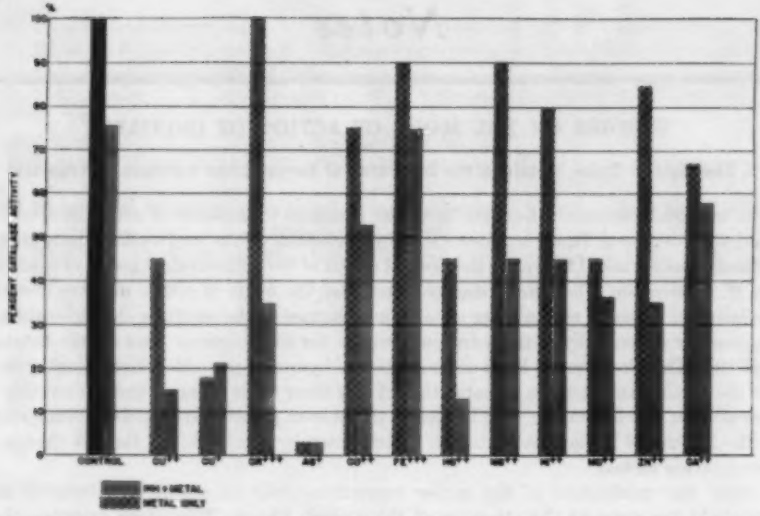

FIG. 1. The effects of various metals on the inhibition of bovine liver catalase by isoniazid. Only the monovalent silver and cuprous ions in this series failed to enhance the inhibition by isoniazid.

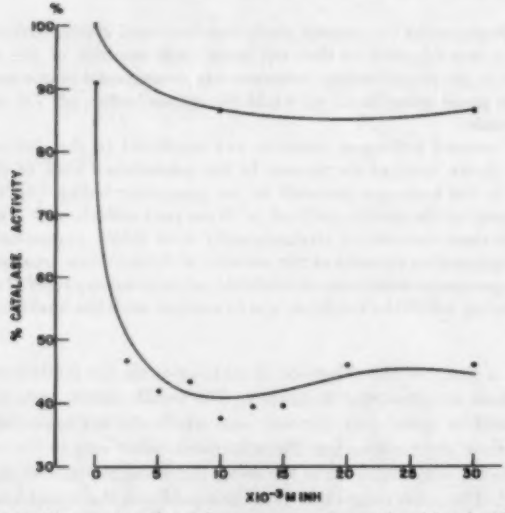

FIG. 2. The effect of increasing the molar concentration ratio of isoniazid to copper on the activity of bovine liver catalase. The upper curve is a control run without adding copper. All points on the lower curve were observed in the presence of 5×10^{-3} M Cu^{++}.

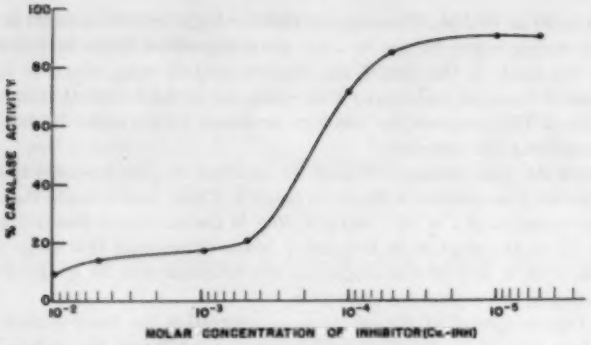

FIG. 3. The effect of an increasing molar concentration of inhibitor (copper chelate of isoniazid) on the activity of bovine liver catalase.

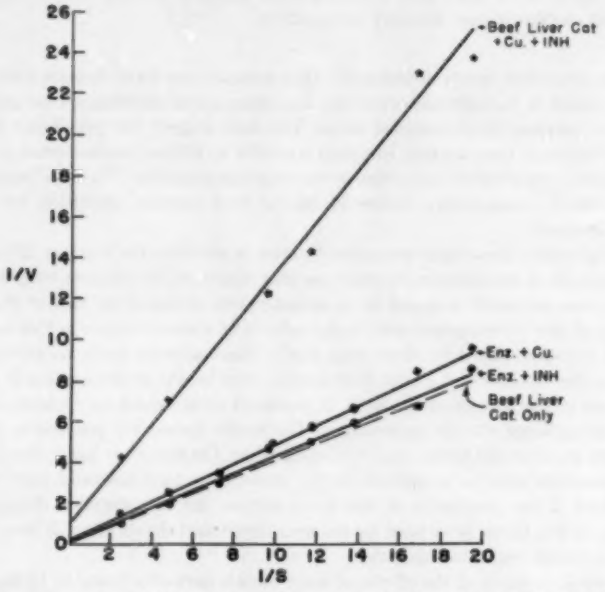

FIG. 4. Lineweaver-Burk plot showing the marked effect of the addition of Cu^{++} on the kinetics of inhibition of bovine liver catalase by isoniazid and demonstrating the competitive nature of this inhibition.

The effect on catalase activity produced by increasing the molar concentration ratio of isoniazid to cupric ion is demonstrated in figure 2. The upper curve in the figure is the control which contained no copper. All of the points on the lower curve were obtained with 5×10^{-3} M copper present as copper sulfate. It may be seen in the upper curve that there was no decrease in catalase activity when the molar concentration of isoniazid was trebled,

viz. from 10 to 30 $\times$ 10^{-3} M. This suggests that the slight inhibition noted in the absence of exogenous copper might be due to trace metal impurities in the reagents. The same observation was made in the case of the stippled control value shown in figure 1. The concentrations of isoniazid and copper were equimolar at the inflection point of the lower curve in figure 2. This supports the structure proposed for the metal chelate of isoniazid by Cymerman-Craig and associates.[4]

The effect of the molar concentration of the inhibitor (copper-isoniazid chelate) on the activity of bovine liver catalase is shown in figure 3. These data indicate that between an inhibitor concentration of 5 $\times$ 10^{-4} and 5 $\times$ 10^{-5} M the reaction is first order. Above and below these limits the reaction is zero order. Since throughout this study an inhibitor concentration of 5 $\times$ 10^{-3} M was employed, the inhibitor was always present in excess and the reaction in this regard was zero order.

In figure 4 the reciprocal of the substrate concentration has been plotted against the reciprocal of the velocity as described by Lineweaver and Burk.[5] These data demonstrate the competitive nature of the inhibition of catalase by copper, isoniazid, the copper-isoniazid chelate, since all of the slopes have a common intercept. It is also quite evident from these data that copper and isoniazid alone are very weakly competitive while the Cu-isoniazid chelate is very strongly competitive.

* * *

The data presented clearly demonstrate that isoniazid can form chelates with ten metals normally present in biologic materials and that these metal chelates are far more efficient inhibitors of catalase than isoniazid alone. The data suggest the possibility that, in the complete absence of trace metals, isoniazid is unable to inhibit catalase since no effort was made to control trace metal impurities in the reagents employed. The data further demonstrate the strictly competitive nature of bovine liver catalase inhibition by the copper chelate of isoniazid.

A question which these data immediately raise is whether the kinetics of inhibition of catalase from *M. tuberculosis* is the same as that observed for catalase from bovine liver. The writers are presently engaged in an investigation designed to answer this question. The results of this investigation will be the subject of a second paper in this series.

Another question raised by these data is why there appears to be no adverse effect of isoniazid on the catalase of the host. Perhaps this may be due to the manner in which trace metals occur in the tissues of the host. If isoniazid were unable to compete successfully with the endogenous chelate molecules of the host's tissues for possession of the trace metals, then there would be no inhibition of catalase. On the other hand, the trace metals in *M. tuberculosis* may be so related to the protoplasm that isonizaid may successfully compete with it for possession of the trace metals. An investigation designed to test the validity of this thesis is planned for the near future and the results will form the subject matter of a future paper in this series.

In summary: A study of the effects of trace metals normally found in biologic material on the inhibition of bovine liver catalase by isoniazid has been described. Ten of twelve metal ions studied markedly enhanced the inhibition. The two which showed no enhancement were the only two monovalent ions in the series. The molar ratio of copper to isoniazid which proved optimal for inhibition was 1:1. Between 5 $\times$ 10^{-4} and 5 $\times$ 10^{-5} M concentration of inhibitor, the inhibition is first order. Above and below these limits it is

[4] Cymerman-Craig, J., *et al.*: Nature, 1956, *177*, 480.

[5] Lineweaver, H., and Burk, D.: J. Am. Chem. Soc., 1934, *56*, 658.

zero order. A kinetic analysis of the inhibition demonstrated its strongly competitive nature in the presence of exogenous copper.

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COMPULSORY ISOLATION OF THE UNCOOPERATIVE TUBERCULOUS PATIENT

The Experience in the State of Georgia

In recent years a great deal of interest has been shown in the compulsory isolation of uncooperative tuberculous patients who will not observe isolation voluntarily. Between 1953 and 1955, 14 states enacted legislation for the compulsory isolation of this recalcitrant group.[1]

Like most states, Georgia has had a control law; but, prior to 1953, anyone unfortunate enough to have tuberculosis who did not observe satisfactory isolation voluntarily was sentenced and made to serve his time in one of the medical sections within our penal system. In other words, he acquired a criminal record and was housed with all types of criminals. Because of the fact that uncooperative tuberculous patients were handled like criminals, there was a great deal of ill feeling toward the old law. It was not used by many of our health authorities except as a last resort and often not at all. A great deal of thought was given to enactment of legislation to manage this group of unfortunates in a more democratic and Christian manner and with less stigma attached. After consultation with phthisiologists and public health authorities, Georgia legislators enacted House Bill No. 802 during their 1953 November–December Session.[2]

The Georgia law states very clearly that *only* a person having, or suspected of having, tuberculosis who does *not observe* proper isolation *voluntarily* may be committed.[3] It also states that such rules and regulations shall be promulgated by the State Board of Health as are necessary to protect the public health against contagious tuberculosis and for the necessary isolation of persons known to have contagious tuberculosis.

The rules and regulations, among other things, very clearly state that the purpose of this act is to *prevent* the spread of tuberculosis *and not* to force hospitalization *or* to make people who have tuberculosis undergo treatment.[3] They further state that, under this law, any person who refuses to clear up the matter of whether or not he is contagious, if his conduct is such as may result in tuberculous infection of other people, may be committed.

In Georgia, therefore, any person with sputum positive for tubercle bacilli who by his action endangers the health of other people may be committed. However, such a person is first given instructions, and in writing, covering the measures that he must carry out to prevent infection of others.[4]

If he violates the instructions so as to expose others, then the local health officer may notify the State Division of Tuberculosis Control of his intention to initiate commitment proceedings (a copy of which is sent to Battey State Hospital); but he must first obtain confirmation of the availability of a bed for such a patient *before* initiating such proceedings.[2, 3] The health officer then files a petition for commitment in the Superior Court

[1] Report on Compulsory Isolation in the United States, December 1, 1955, by the Social Research Division of the National Tuberculosis Association.

[2] Control Act approved December 22, 1953 (Georgia Laws 1953 November–December Session, page 348).

[3] Rules and Regulations Formulated by the State Board of Health of Georgia as required by Tuberculosis Control Act. H.B. No. 802. (approved by State Board of Health May 20, 1954).

[4] Memoranda by Division of Tuberculosis Control, Georgia Department of Public Health, June 24, 1954, August 13, 1954, October 14, 1954, December 1, 1954, and March 15, 1955.

in the county where the person resides.[2] A copy of the petition and order is served on the person named in the petition.[2] The hearing is held in five to fifteen days and the results of the hearing are made known to the State Division of Tuberculosis Control and Battey State Hospital.[2]

Our commitment order reads in part:

> The patient shall be confined in Battey State Hospital, or such other hospital approved by the State Board of Health, *until* such time as the superintendent thereof determines that the patient no longer has contagious tuberculosis, *or* that his discharge will not endanger public health.[3]

For purposes of this act, it has been agreed that "noncontagious" shall mean having six consecutive months of cultures negative for tubercle bacilli of sputum and/or gastric washings, or of such other secretions as were positive for tubercle bacilli.

All committed patients are admitted directly to the locked section of the commitment wards.[3] Prior to May, 1956, there was no maximal security ward, which resulted in a fair percentage of escapees. Since May, 1956, this has not been a problem. Upon admission every patient has the same medical examination, has his case considered in the same way at the staff conference, and is given precisely the same treatment as he would have had as a free voluntary admission. His nursing, medical, surgical, and all other management is *no* different from that of any free patient.

The main objective, apart from rendering him noncontagious, is rehabilitation; and, therefore, after a period of individual and group counseling, in which every effort is made to re-direct his thinking and his efforts, if he shows that he may be trustworthy, his case is discussed before a Board consisting of five doctors. He is considered for transfer to an open free ward, where he remains and is treated like any other free patient except that he has no pass privileges. (In the 1957 Legislature, provisions were made to grant emergency and therapeutic leaves under certain conditions.)[5] If the patient becomes maladjusted while on the free ward, he is again returned to the locked section for further counseling and guidance, but only with the approval of the chief of that service.[3] He may again be considered for the free ward when he is adjusted, and this procedure may be repeated several times. Every effort is made toward solving the many problems that the average recalcitrant patient has; and the services of the chaplain and Social Service Department are freely sought and used. The objective is *always* to render him noncontagious *and* to rehabilitate him.

If and when a patient reaches the point of noncontagion, whether or not his tuberculosis is still active, his commitment is terminated, and all interested parties are so notified.[3] The patient is encouraged to remain in the hospital until he qualifies for, and obtains, a medical discharge. At this writing no committed patient so released has had to be re-committed, and the irregular discharge rate of those who have had their commitments terminated compares favorably with that among our white male patients in general.[6]

Between September 10, 1954, and September 1, 1956, 64 white males, 7 white females, 15 Negro males, and 6 Negro females (a total of 92 patients) were admitted to Battey State Hospital under the new commitment law (table 1). Of these 92 patients, 71 (77.2 per cent) had far advanced tuberculosis and 73 (79.3 per cent) had sputum positive for tubercle bacilli on culture; but only 66.3 per cent had sputum positive for tubercle bacilli on microscopy (table 2). Twenty per cent of these 92 patients were found to be noncontagious and were, of course, released from commitment promptly.

[5] Act No. 234 (Senate Bill No. 12), approved March 7, 1957 (Georgia Laws 1957 Session).
[6] Annual Reports, Battey State Hospital, 1955 and 1956.

TABLE 1

92 Patients Committed by Year, Sex, and Race

Year	W-M	W-F	N-M	N-F	Total
1954	15	2	0	0	17
1955	30	2	4	0	36
1956	19	3	11	6	39
Total	64	7	15	6	92

TABLE 2

Bacteriologic Status at Admission

	W-M	W-F	N-M	N-F	Total
Number of smears	40.0	6.0	10.0	5.0	61.0
Per cent of smears	62.5	85.7	66.6	83.3	66.3
Number of cultures	53.0	6.0	9.0	5.0	73.0
Per cent of cultures	82.8	85.7	60.0	83.3	79.3

TABLE 3

Average Age and Admission Classification

	W-M	W-F	N-M	N-F
Age	46 (30–70)	37 (30–45)	43.6 (31–63)	28.6 (23–39)
Far advanced	48	7	12	4
Moderately advanced	14	0	2	1
Minimal	2	0	1	1

TABLE 4

Number of Previous Admissions and Irregular Discharges

	W-M	W-F	N-M	N-F
Number of previous hospitalizations	188	31	45	15
Number of unsanctioned discharges	165	26	33	9
Average number of unsanctioned discharges	2.56	2.56	2.02	1.5

TABLE 5

Prison, Alcoholic, and Narcotic Record of 64 White Male Committed Patients

	Number	Per Cent
Total prison record	54	84.40
Felony (plus)	12	18.75
Misdemeanor	42	65.60
Alcoholism	42	65.60
Addiction	3	4.70

TABLE 6

DISCIPLINARY PROBLEMS AND DISPOSITION OF 64 WHITE MALE COMMITTED PATIENTS

64 have had one or more trials on an open free ward.
33 (51.5%) have had disciplinary problems 53 times.
12 have had surgery.
All have had chemotherapy.
48 have converted sputum by culture for 2 months or longer.
30 (46.8%) have had their commitment terminated.
12 of these 30 had unsanctioned discharges (A.M.A. rate 40%).
30 are still in the hospital (sputum of 15 still positive for tubercle bacilli on smear).

TABLE 7

TUBERCULIN REACTIONS OF 208 HOUSEHOLD CONTACTS TO 92 COMMITTED PATIENTS

	Number Tuberculin Tested	Number of Positive Reactors	PerCent Positive Reactors
To 92 committed patients	208	118	56.7
In Muscogee County survey	394	161	40.9
Beeuwkes, Hahn, and Putnam	743	590	79.4

TABLE 8

ROENTGENOGRAPHIC RESULTS OF 258 HOUSEHOLD CONTACTS TO 92 COMMITTEE PATIENTS

	Numbered Examined Roentgenograghically	Number of Roentgenograms Showing Tuberculosis	Percentage of Roentgenograms Showing Tuberculosis
To 92 committed patients	258	10 active 3 suspicious 1 primary, arrested	4.6 active 5.4 total
Beeuwkes, Hahn, and Putnam	987	439	38.8

Except for the Negro females, the recalcitrant group approached middle age. The average age was 43.8 years and the ages varied from 23 to 70; likewise, except for the Negro females, all patients had had at least two previous unsanctioned discharges before being committed (average 2.53) (tables 3 and 4).

White males are generally accepted as the worst management problem and, since this was the only group large enough (64 patients) to contribute reasonably accurate percentages, it was used to present some of the results. Fifty-four (84.4 per cent) of the white males had prison records, 42 (65.6 per cent) had a history of alcoholism, and 3 were known addicts (table 5). The addicts presented a serious problem in management, but the alcoholics were the most difficult to rehabilitate, and 51.5 per cent of them presented disciplinary problems. Despite the management problems with the 64 white male patients, 30 (46.8 per cent) became noncontagious and had their commitment terminated; and only 12 of these 30 had subsequent unsanctioned discharges (table 6). The unsanctioned discharge rate of 40 per cent compares favorably with the over-all unsanctioned discharge rate of the free white male patients in Battey State Hospital, which was 44 per cent in 1955 and 43.1 per cent in 1956.

Having observed that very few persons were admitted to Battey State Hospital with a history of exposure to our committed patients, an epidemiologic study of 208 household contacts of the 92 committed patients was made (tables 7 and 8). In comparing these results with those of a large recent study in Muscogee County, Georgia, and a larger older study in the states of New York and Pennsylvania, it was noted that there were 16 per cent more positive tuberculin reactors in the present group than in the Muscogee County group, but 20 per cent fewer than in the New York and Pennsylvania groups. There were one-seventh as many new cases of tuberculosis in the contacts in the present group as in the New York and Pennsylvania contacts[8] (5.4 per cent compared with 38.8 per cent).

In Georgia a compulsory isolation law has been found to be very useful in controlling the uncooperative tuberculous patients, who apparently do not cause as many new cases of tuberculosis as one would have suspected. Despite the fact that there is a high incidence of alcoholism and prison background among the patients referred to herein, the law has proved that with adequate management and training many of these patients can be restored to a noninfectious sputum status and returned to society as noncontagious and perhaps also as more desirable individuals.

BATTEY STATE HOSPITAL
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May 27, 1957

[7] 1955 Report of Survey from Muscogee County, Georgia.

[8] BEEUWKES, H., HAHN, R. G., AND PUTNAM, P.: Am. Rev. Tuberc., 1942, *45*, 165.

EVALUATION OF THE CHICK YOLK SAC METHOD AS COMPARED WITH CONVENTIONAL LABORATORY PROCEDURES FOR THE ISOLATION OF MYCOBACTERIUM TUBERCULOSIS

Although previous reports[1-10] suggested the embryonated hen's egg as a specific and sensitive medium for rapid isolation of *M. tuberculosis*, it was believed to be advisable to compare the application of this newer technique with conventional methods in the routine diagnostic laboratory.

The materials came from patients in Charity Hospital of Louisiana at New Orleans whose illnesses resembled some form of tuberculosis, but whose disease remained undiagnosed after conventional studies in hospital laboratories.

Sputum, bronchial washings, and gastric washings were concentrated by centrifugation after digestion with either trisodium phosphate or sodium hydroxide neutralized with hydrochloric acid. Penicillin and chloramphenicol were added to the concentrates to give approximately 10 units and 10 γ respectively, per ml. to reduce bacterial contamination. Cerebrospinal fluid, pleural fluid, and other uncontaminated specimens were concentrated by centrifugation. Biopsy material was triturated to give a 10 per cent suspension in saline or saline-antimicrobial drug solution. The yolk sacs of three or four seven-day-old embryonated chicken eggs were then inoculated with 0.1 to 0.5 ml. each of the concentrate or triturate, the exact volume depending on the total amount of material available after preparation. At the same time three tubes of Lōwenstein-Jensen medium were inoculated with a similar volume of the prepared material. The eggs were incubated at 37°C. and all embryos that died within the first seventy-two hours were discarded. Incubation of all remaining embryonated eggs was continued until death or seven days following inoculation, whichever occurred sooner. The yolks were then harvested and prepared for further studies. Four-day-old embryos were found unsuitable, principally because of the high mortality.

Although the method employed for handling the yolk was much the same as that outlined by Brueck and Buddingh[8, 9] it was found to be advisable to introduce two modifications. A considerable variation was noted in the number of mycobacteria which could be found in successive samples from the same infected yolk. The variability appeared in both direct smears and water-phenol flotation concentrates. This suggested aggregate or colonial growth of *M. tuberculosis* within the yolk. Consequently, yolks from all harvested eggs that had been inoculated from a single clinical specimen were pooled and blended in a sterile semi-micro, Monel metal, Waring Blendor.® This dispersed the bacilli more uniformly. In order to reduce the danger of infectious aerosol, the top of the blendor was tightly sealed with parafilm, and the blendor was not opened for thirty minutes following blending. The blended yolk was removed with a sterile pipette. Smears were stained by Darrow's modification of the Ziehl-Neelsen method[11] using light green SF as a counterstain. At the same time, 0.1 ml. of the blended yolk was inoculated onto two slants of Lōwenstein-

[1] Goodpasture, E. W., and Anderson, K.: Am. J. Path., 1937, *13*, 149.
[2] Costil, L., and Bloch, F.: Compt. rend Soc. de Biol., 1938, *128*, 40.
[3] Moore, M.: Am. J. Path., 1942, *18*, 827.
[4] Emmart, E. W., and Smith, M. I.: Pub. Health Rep., 1941, *56*, 1277.
[5] Dubos, R. J., *et al.*: Am. Rev. Tuberc., 1946, *54*, 204.
[6] Lee, H. F.: Ann. New York Acad. Sc., 1949, *52*, 692.
[7] McNelly, E., and Riddell, W. A.: Am. J. Pub. Health, 1949, *39*, 1463.
[8] Brueck, J. W., and Buddingh, G. J.: Proc. Soc. Exper. Biol. & Med., 1952, *80*, 589.
[9] Buddingh, G. J., and Brueck, J. W.: J. Louisiana State Med. Soc., 1953, *105*, 304.
[10] Bunn, P., *et al.*: J. Lab. & Clin. Med., 1955, *45*, 658.
[11] Darrow, M. A.: Stain Tech., 1949, *24*, 93.

Jensen medium and, frequently, into two tubes of Dubos Tween®-albumin broth. Finally, 1.0 ml. of the blended yolk was inoculated in the thigh muscle of a guinea pig. The second modification facilitated the concentration of the blended yolk. A dextrose solution having a specific gravity of 1.030 was substituted for the distilled water used by Brueck and Buddingh for emulsifying the yolk sample. A 3 ml. sample of the blended yolk was placed in a 50 ml., wide-mouth, centrifuge tube. Sterile dextrose solution of specific gravity 1.030 was added to fill half the tube. The tube was then closed with a rubber stopper and shaken vigorously. Five milliliters of pure phenol were next added, and the tube was stoppered and shaken again. Finally, more dextrose solution was added to fill the tube, and it was covered and allowed to stand for two hours. Smears were made from the fat-phenol layer on the surface with either a loop or a capillary pipette. After drying, the smears were treated with ether to remove the fat and were then stained by Darrow's method.[12]

Four slides were prepared from both the concentrated and the unconcentrated yolk. Each slide was examined three times across the smear under the oil immersion lens before being reported as negative for *M. tuberculosis*. The Löwenstein-Jensen and Dubos broth subcultures of the yolk were incubated eight weeks and inspected at two-week intervals. Guinea pigs were examined either at autopsy at death or when sacrificed at eight weeks after inoculation.

Contaminants appeared frequently in the harvested yolk despite treatment of specimens with either sodium hydroxide or trisodium phosphate and the addition of antimicrobial drugs prior to inoculation. For this reason the blended yolk was subcultured on blood agar plates. By contrast, sodium hydroxide or trisodium phosphate digestion was usually sufficient to prevent significant contamination of the dye-containing solid Löwenstein-Jensen slants. The identification of contaminants was found advisable because some of these closely resembled tubercle bacilli after growing in the yolk sac. Thus, staphylococci, diphtheroids, and certain bacilli were acid fast in the Darrow-stained yolk smears, and the latter two groups assumed a morphology resembling yolk-nurtured *M. tuberculosis*. When such contaminants were recovered on blood agar plates and acid-fast forms were seen on smears, it was necessary to report that yolk as "questionably positive."

Ninety-four clinical specimens were tested by the embryonated egg-yolk-sac method and conventional solid media. The results according to the type of specimen submitted to the laboratory are summarized in table 1. Premature death of chick embryos was a rather frequent occurrence, and such specimens had to be excluded from the comparative study. This difficulty will be discussed later.

An embryonated egg was considered positive for *M. tuberculosis* when guinea pigs inoculated intramuscularly with the yolk developed tuberculosis. Although the yolk material was always cultured on solid medium and about one-third of the specimens in liquid medium as well,[12] there was no instance in which the yolk was positive by culture and negative by guinea pig.

There appears to be little difference in the sensitivity of conventional methods of culture and the chick embryo yolk sac for recovering *M. tuberculosis* from the clinical specimens. The isolation of tubercle bacilli from the yolk sac alone in six instances as compared with the four isolations on solid medium only is not statistically significant. It is evident, however, that the total percentage of positive diagnoses was significantly increased by utilization of the embryonated egg.

Next, the microscopic study of stained smears of both concentrated and unconcentrated yolk was compared with the findings in guinea pigs inoculated with the same yolk. Microscopic interpretations of stained smears were surprisingly difficult. Preliminary trials with sputum concentrates containing tubercle bacilli as inocula revealed clumps of typical mycobacteria after incubating embryonated eggs six to seven days. After only three or

[12] Dubos, R. J., and Middlebrook, G.: Am. Rev. Tubere., 1947, *56*, 345.

TABLE 1

COMPARISON OF THE SENSITIVITY OF THE CHICK EMBRYO YOLK SAC AND SOLID CULTURE MEDIUM FOR ISOLATION OF M. TUBERCULOSIS USING VARIOUS SPECIMENS

Specimens		Number Positive by:			Total Positive
Group—Type	Number	Yolk Sac Only	Solid Medium Only	Both Methods	
I. Sputum Gastric washing Bronchial washing Lung biopsy	24	1	0	5	6
II. Abscess Subcutaneous nodule	11	1	0	3	4
III. Lymph Node Bone marrow	13	1	2	1	4
IV. Serous Fluid Cerebrospinal fluid	46	3	2	2	7
Totals	94	6	4	11	21

four days of incubation, however, many of the tubercle bacilli appeared singly and as very short rods which stained evenly and less densely than those in the yolk after six days of incubation. Clumps of tubercle bacilli were found infrequently in microscopic examination of yolk, concentrated or unconcentrated, that had been inoculated with the specimens used in this study. Indeed, there were many yolk smears that contained only one or two acid-fast rods, presumably due to the small number of bacilli in the inoculum or to the short duration of incubation of the infected eggs. When many such acid-fast rods were found, beaded or unbeaded, the examiner always suspected the presence of contaminants. Although blood agar plates, which were inoculated with the yolk material, usually confirmed the presence of contaminants, these failed to exclude the presence of tubercle bacilli. Thus, it was necessary to report many smears as questionably positive. This problem appeared to be greater for the interpretation of stained concentrates of yolk than for stained smears of yolk. Of the ninety-four specimens, questionably positive *concentrates* were reported for twenty-four and questionably positive single *smears* for five of the blended yolks recorded as "positive" in the construction of table 2.

In table 3 the results of the subculture of yolk material onto solid and liquid media and the inoculation of the same material into guinea pigs are compared. Only 40 per cent of the yolks positive for *M. tuberculosis* by guinea pig inoculation were positive when subcultured onto Löwenstein-Jensen slants. The 0.1 ml. of the yolk material used in seeding the slant formed a tenacious and glazed deposit over the surface of the solid medium, much as it does on glass surfaces. Acid-fast bacilli were occasionally found in smears of the scraped surfaces of Löwenstein-Jensen slants when no colonial growth could be observed. The poor correlation between the results of inoculating guinea pigs with yolk and the subculture of a portion of the yolk onto Löwenstein-Jensen medium was thought to be due to the poor environmental conditions produced by the glazed and gummy yolk on the slant's surface. However, the report of Bunn and his co-workers[10] makes this explanation unlikely. They experienced no difficulty in obtaining successful

TABLE 2

COMPARISON OF SENSITIVITY OF CULTURE MEDIA WITH GUINEA PIG INOCULATION FOR ISOLATING M. TUBERCULOSIS FROM INFECTED CHICK EMBRYO YOLK

	Positive by Guinea Pig Inoculation		
	Number Tested	Number Positive	Per Cent False Negative
Solid medium	15	6	60
Liquid medium	5	3	40
	Negative by Guinea Pig Inoculation		
	Number Tested	Number Negative	Per Cent False Positive
Solid medium	73	73	0
Liquid medium	26	26	0

TABLE 3

COMPARISON OF STAINED SMEARS WITH GUINEA PIG FINDINGS FOR IDENTIFYING M. TUBERCULOSIS IN YOLK OF INFECTED CHICK EMBRYO

	Positive by Guinea Pig Inoculation		
	Number Tested	Number Positive	Per Cent False Negative
Concentrated yolk smear	17	13	23
Unconcentrated yolk smear	12	4	66
	Negative by Guinea Pig Inoculation		
	Number Tested	Number Negative	Per Cent False Positive
Concentrated yolk smear	75	44	41
Unconcentrated yolk smear	65	54	17

culture by planting the surface of Americad Trudeau Society medium slants[12] with 1.0 ml. of yolk. As, in the present study, 1.0 ml. of yolk was used for inoculating the guinea pigs, and only 0.1 ml. of yolk for seeding the Löwenstein-Jensen slants, it is probable that the difference in the number of yolks positive by animal inoculation and the number positive by subculture onto solid slants was purely a reflection of the volume inoculated. If this is the true explanation of the poor results from subculturing yolk to solid slants, it appears probable that 60 per cent of the positive yolks contained very few organisms per milliliter of yolk. This correlates well with the fact that many of the yolk smears revealed only one or two acid-fast rods.

* * *

The yolk sac of an embryonated hen egg provides a suitable medium for the multiplication of tubercle bacilli. After six or seven days of incubation, the smeared and stained yolk will reveal acid-fast bacilli during careful microscopic search if the original inoculum contained as few as 10 to 100 mycobacteria.[10] Unfortunately, certain commonly encountered contaminating organisms and, perhaps, some artefacts, present a morphology and

[12] AMERICAN TRUDEAU SOCIETY: Report of Committee on Evaluation of Laboratory Procedures, Am. Rev. Tubere., 1946, *54*, 428.

staining characteristics that closely resemble mycobacteria. This fact accounted principally for the intolerable percentage of false positive microscopic readings. If microscopic examination of the stained yolk cannot be relied upon for even tentative diagnosis, then the method loses any advantage of speed. In the writers' experience the presence of artefacts and contaminants closely resembled young forms of *M. tuberculosis* in yolk, and gave rise to a high percentage of false-positive smears. As a consequence, verification was necessary by either subculturing yolks onto conventional solid medium slants or by inoculating guinea pigs. In the routine use of Löwenstein-Jensen medium in this laboratory, colonies of *M. tuberculosis* are usually found after fourteen to twenty-one days of incubation. If subculture of the yolk onto solid media required a minimum of ten days' incubation[10] for recognizable growth, the yolk-sac method would have failed to shorten the time required for the specific diagnosis of tuberculosis. Thus, the use of the chick embryo yolk sac for recovering tubercle bacilli from the clinical materials submitted for diagnosis was disappointing. Since the method succeeded six times when conventional solid medium failed, and failed four times when the solid medium succeeded, the total number of isolations was increased by use of two methods. It seems possible that as much could have been achieved by substituting additional Löwenstein-Jensen slants for embryonated eggs.

There were certain important considerations dealing with the utility of the yolk sac method which do not appear in the present results. The first of these was premature death of the embryos. Eggs which died before the end of the third day of incubation were usually contaminated by gram-negative rods such as members of the Pseudomonas genus. These eggs were not useful for further study and were discarded. In this study, 26 per cent of all eggs inoculated were lost due to premature death. Loss of one or two eggs out of four was not unusual when specimens such as sputum were employed. Occasionally, as many as three of the four eggs were lost. The second major consideration was connected with the first but involves the seasonal availability and usefulness of embryonated chicken eggs. Embryonated eggs were scarce as well as fragile during the summer months in semitropical New Orleans; thus, yolk sac studies were impossible during three months of the year.

In summary: Although the yolk sac of the chick embryo provides a nutritional environment which permits rapid growth of *Mycobacterium tuberculosis*, it seems best suited to studies of mycobacteria from pure culture. The morphology and staining qualities of other organisms, which were introduced into the yolk sac when clinical materials were used for diagnostic purposes, frequently simulated the variable morphology and tinctorial properties of *M. tuberculosis* in yolk, making a diagnosis by stained smears of yolk unreliable. Acid-fast bacilli in the yolk sac of embryonated chicken eggs that were inoculated with concentrates of clinical specimens required verification by animal inoculation or subculture on conventional solid media before a diagnosis of tuberculosis could be established. For this reason, the yolk-sac method did not result in any saving of time necessary for diagnosis. The yolk sac of embryonated chicken eggs appeared to be equally as sensitive as conventional laboratory media for the isolation of *M. tuberculosis*. There were additional disadvantages of the method, such as expense and the seasonal variation in viability of embryos, not inherent in conventional laboratory methods for the isolation of *M. tuberculosis*.

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A CURRENT ANALYSIS OF TUBERCULOSIS DEATHS IN NEW YORK CITY

It is well recognized that there has been a marked decline in the tuberculosis mortality rate in recent years—a rate which, in New York City, reached a low of 12.0 deaths per 100,000 population in 1956, compared with a rate of 70.4 in 1931. As a consequence, there is an increased interest at present in knowing not only *how many* persons die each year of tuberculosis but *why* and *how* they die in the face of intensive case finding and the availability of effective chemotherapy. In order to determine the feasibility of using data derived from death certificates for this purpose, an analysis was made of a sampling of death certificates in which tuberculosis was listed as the underlying cause of death.

During April, 1956, all death certificates in which tuberculosis was listed as the underlying cause of death were collected consecutively. Simultaneously, death certificates were analyzed on all persons whose names were removed from the active tuberculosis roster during this same period because of death due to nontuberculous causes. In addition, those death certificates processed through the Bureau of Tuberculosis during April, 1956, which listed previously unregistered active tuberculosis as a contributory cause of death or as an incidental finding were also studied. Because of technical difficulties, no attempt was made to determine how many deaths ascribed to nontuberculous causes in persons whose names were not on the active roster might have been due to or associated with active tuberculosis.

During the study period 100 death certificates were collected which listed tuberculosis as the underlying cause of death. The age, race, and sex distribution among this group of cases was essentially the same as the distribution for the entire group of persons certified as having died of tuberculosis in New York City during 1956. Adequate information was also available for 26 persons whose names were removed from the active tuberculosis roster because of death from causes other than tuberculosis. In two instances, the death certificates indicated that active tuberculosis was an incidental finding at death in a person whose name had never been registered with the Bureau of Tuberculosis.

Information on the cases studied was obtained from clinic and hospital records, autopsy reports when available, medical examiner's reports, and a review of available chest roentgenograms. In most instances, considerably more information (including necropsy findings) was available to the writer than to the physicians who filled out the death certificates. Death certificate diagnoses were corrected by the writer only if there was unequivocal evidence supporting such a change; otherwise, the diagnoses, as listed on the certificate, were accepted for the purposes of this study, even though, in some instances, there was considerable doubt as to their accuracy. Of the 100 cases in which tuberculosis was given as the underlying cause of death, 14 were certified by a medical examiner, usually without the benefit of an autopsy or adequate clinical or laboratory data. Autopsies were performed in 26 of these 100 cases, but the final anatomic findings were not always available at the time of certification. Corrections of death certificates which were issued prior to availability of necropsy findings *were not usually made* by the certifying physician even when the final anatomic diagnoses were entirely at variance with those made at the time of death certification.

The distribution of the 128 death certificates analyzed is found in table 1.

Only 64 of the 100 patients with tuberculosis certified as the underlying cause of death had clinical or autopsy findings that justified this certification. In 36 instances, the underlying cause of death was thought by the present writer to be nontuberculous; of these, 15 patients had clinical or pathologic evidence of active tuberculosis at the time of death, while 21 did not. In the former group of cases, active tuberculosis was found as an incidental finding in association with the following underlying causes of death: cardiovascular disease not secondary to pulmonary tuberculosis, 7 cases; metastatic carcinoma,

TABLE 1

DISTRIBUTION OF 128 DEATH CERTIFICATES COLLECTED FOR ANALYSIS DURING APRIL, 1956
Bureau of Tuberculosis, New York City Department of Health

Category	Number			
Tuberculosis certified as underlying cause of death	100			
Tuberculosis confirmed as underlying cause of death		64		
Not previously registered			21	
Died within 2 years of previous registration			12	
Died 2 years or more after previous registration			31	
Tuberculosis not confirmed as underlying cause of death		36		
Active tuberculosis present at time of death			15	
Not previously registered				4
Previously registered				11
Active tuberculosis not present at time of death			21	
Not previously registered as tuberculosis				9
Previously registered as tuberculosis				12
Nontuberculous diseases certified as underlying cause of death	28			
Certified diagnoses confirmed		28		
Not previously registered as having tuberculosis			2	
Active tuberculosis certified as incidental finding				2
Previously registered as having tuberculosis and still on active roster at time of death			26	
Active tuberculosis listed on death certificate				7
Inactive tuberculosis listed on death certificate				13
No mention of tuberculosis on death certificate				6
Certified diagnoses not confirmed		0		

TABLE 2

DISTRIBUTION OF 36 DEATH CERTIFICATES WHICH LISTED PREVIOUSLY UNREGISTERED ACTIVE TUBERCULOSIS AS PRESENT AT TIME OF DEATH

Diagnoses as Given on Death Certificate	Corrected Diagnoses after Review	Number	Per Cent
Total number of death certificates which listed previously unregistered active tuberculosis as present at time of death		36	100.0
Tuberculosis considered underlying cause of death	Same	21	58.3
Tuberculosis considered underlying cause of death	Nontuberculous diseases considered underlying cause of death; active tuberculosis present as incidental finding at time of death	4	11.5
Tuberculosis considered underlying cause of death	Nontuberculous diseases considered underlying cause of death; active tuberculosis not present at time of death	9	25.0
Nontuberculous diseases considered underlying cause of death; active tuberculosis listed as incidental	Same	2	5.6

3 cases; nephrosclerosis and uremia, one case; syringomyelia, one case; acute toxic hepatitis, one case; acute intestinal obstruction, one case; and a perforated gastric ulcer in one case.

In the 2 previously unregistered cases in which active tuberculosis was certified as a minor contributory cause of death, the diagnoses, as given on the death certificate, were substantiated by the clinical findings.

Of the 26 cases removed from the active tuberculosis roster for deaths ascribed to causes other than tuberculosis, 7 patients were certified as having active tuberculosis as an incidental finding at the time of death; 13 were certified as having inactive tuberculosis; and in 6 instances, no mention of tuberculosis was made on the death certificate. In no instance did the evidence implicate tuberculosis as the main cause of death.

* * *

In the present sample, there were 36 instances in which active tuberculosis was registered for the first time at death. The distribution of these cases is given in table 2. Nine of these patients (25 per cent) were considered by the writer to have had no evidence of tuberculosis at the time of death.

* * *

Of the 64 cases in which tuberculosis was confirmed as the underlying cause of death, 43 had significant complications as follows: 13 patients suffered from chronic alcoholism, 11 had been psychotic or severely emotionally disturbed prior to death; 12 had cardiovascular disease; 4 had diabetes mellitus; 2 died as a result of postoperative complications following pulmonary resection; and one patient had an advanced stage of scleroderma, In addition to these primary complications, compiled on the basis of listing only the most important complication when more than one occurred, were many secondary complications, e.g., 2 patients had diabetes mellitus in addition to other major complications, and 6 patients had chronic alcoholism in addition to other more important complications.

In summary: A sample analysis of tuberculosis deaths from death certificates revealed that, although this type of study does give information that could prove useful in tuberculosis control, such an analysis might prove to be misleading without further investigation to substantiate the certified cause of death. In the present study, only 64 of the 100 deaths certified as being primarily due to tuberculosis were found to be based on reasonably good clinical or pathologic grounds. On the other hand, after a careful review of the available records, none of the certified nontuberculous deaths which occurred in a group of 26 persons whose names were on the active tuberculosis roster at the time of death could be ascribed to tuberculosis.

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SOME GENETIC CONSIDERATIONS ON THE ISONIAZID-RESISTANCE SYSTEM OF MYCOBACTERIUM AVIUM

The occurrence of isoniazid-resistant variants in *Mycobacterium tuberculosis* not previously exposed to isoniazid has been demonstrated by Karlson and Ikemi,[1] and the result suggests that isoniazid-resistant variants are derived from spontaneous mutation. Genetic information concerned with isoniazid resistance of mycobacteria is lacking at present. It is the purpose of this paper and a companion paper on the isoniazid resistance system of *Mycobacterium tuberculosis* var. *hominis*[2] to present genetic studies on isoniazid resistance.

Mycobacterium avium, strain Jucho, was used throughout the study.

Modified Sauton medium and modified Sauton agar medium were used. The composition of the modified Sauton medium is as follows: glycerin, 50.0 ml.; citric acid, 2.0 gm.; magnesium sulfate, 0.5 gm.; dibasic potassium phosphate, 0.5 gm.; ferric ammonium citrate, 0.05 gm.; sodium glutamate, 4.0 gm.; distilled water, 950 ml. (The pH of the medium was adjusted to pH 7.0 by ammonia and autoclaved at 115°C. for thirty minutes.) The medium was poured in 50 ml. quantity into 200 ml. Erlenmeyer flasks. Modified Sauton agar medium is the modified Sauton medium containing 3 per cent agar. The medium was poured in 30 ml. quantity into a Petri dish 9 cm. in diameter. The percentage of agar is most important, for the bacteriostatic activity of isoniazid is significantly influenced by the concentration of agar. The higher the concentration of agar, the lower the activity of isoniazid.

Isoniazid[3] was sterilized by Seitz filtration and added to the medium dissolved immediately before use.

The method of testing the number of survivors on various isoniazid concentrations in the total viable bacterial population consisted of inoculating a series of the modified Sauton agar plates containing various isoniazid concentrations with tenfold dilutions of a cell suspension. Each series of plates consisted of 5 plates containing: no drug, 0.1 γ, 0.2 γ, 0.5 γ, 1.0 γ, 2.0 γ, 3.0 γ, 4.0 γ, 5.0 γ, 10.0 γ, 20.0 γ, 50.0 γ, and 100.0 γ of isoniazid per ml. of medium, respectively. The series of plates were inoculated by each 0.1 ml. of 10^0 to 10^{-4} dilution of the cell suspension. The cell suspension was prepared as follows: A seven-day culture of the organism in the modified Sauton medium, which had been inoculated with several hundred viable cells—and, therefore, very probably not inoculated with any mutant—was shaken with glass beads for ten minutes and suspended with saline. The plates were incubated at 37°C. for five days and the number of survivors was calculated on various concentrations of the drug. Counts were made from each series of plates at a dilution of the suspension giving discrete colonies but not less than 50 and not more than 300 per plate.

The number of cells capable of growing on the various drug concentrations was expressed as percentage of the total viable bacterial population.

Colonies of survivors obtained by the foregoing procedure were tested for the composition of bacterial population with respect to isoniazid resistance. A series of colonies grown on plates were selected and shaken with glass beads and suspended with saline. Each 0.1 ml. of appropriate dilutions of the suspension was inoculated onto a series of Sauton agar plates containing various concentrations of isoniazid, and the plates were incubated for five days. Counts were made from a series of plates giving discrete colonies. The composition of the bacterial population with respect to isoniazid resistance was expressed as percentage of survivors grown on medium containing various drug concentrations against survivors grown on medium containing no drug.

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[1] Karlson, A. G., and Ikemi, Y.: Proc. Staff Meet., Mayo Clin., 1952, *27*, 239.

[2] Tsukamura, M., and Noda, Y.: In press.

[3] Manufactured by Shionogi Co.

TABLE 1

RATIO OF THE NUMBER OF SURVIVORS ON VARIOUS ISONIAZID CONCENTRATIONS (Medium: Modified Sauton Agar Medium)

Isoniazid Concentration	Ratio of Survivors
γ *per ml.*	
0	1.00
0.1	1.18
0.2	1.21
0.5	1.10
1.0	0.607
2.0	0.324
3.0	2.25×10^{-4}
4.0	8.85×10^{-5}
5.0	7.75×10^{-5}
10.0	6.74×10^{-5}
20.0	6.04×10^{-5}
50.0	1.86×10^{-5}
100.0	$<1 \times 10^{-7}$

The results are shown in table 1. As shown in the table, isoniazid was not selective for almost all individual cells in the strain at a concentration of 0.5 γ per ml., and it was selective for a number of individual cells at concentrations of 1.0 to 2.0 γ per ml. The number of survivors decreased very markedly at a concentration of 3.0 γ per ml. The number of survivors at 2.0 γ of isoniazid per ml. was 32.4 per cent of the total viable bacterial population and the number at 3.0 γ per ml. was only 0.0225 per cent of the total viable bacterial population (approximately one-thousandth of that at 2.0 γ per ml.). The number of survivors was not significantly influenced by increasing the concentration of isoniazid from 3.0 to 50.0 γ per ml. Survivors capable of growing on medium containing 100.0 γ of the drug could not be found among 10^7 viable cells.

The composition of bacterial population of strains obtained by one-step selection of various isoniazid concentrations—that is, seven-day cultures—obtained by the above experiment was tested. The results are shown in table 2. Among descendants of survivors on medium containing 1.0 γ or 2.0 γ of isoniazid, variants resistant to more than 3.0 γ of isoniazid were not found, or only a few were found. On the other hand, among descendants of survivors grown on medium containing more than 3.0 γ of isoniazid, a large number of variants resistant to more than 3.0 γ of isoniazid were found, and percentages of the variants of the total viable bacterial population were not affected by the drug concentration used for one-step selection. A comparison was made of the composition of population expressed as ratio of variants resistant to 10.0 γ of isoniazid in the total viable bacterial population between a one-step mutant strain obtained by one-step selection of 50.0 γ of isoniazid and a multiple-step mutant strain obtained by ten successive transfers onto media containing 50.0 γ of isoniazid. The results are shown in table 3. The one-step mutant strain represented a significant difference between the number of survivors on medium containing 10.0 γ of isoniazid and that on medium containing no drug, i.e., a heterogeneous composition of population. On the other hand, the multiple-step mutant strain represented no significant difference between them, i.e., a homogeneous composition of population.

* * *

The culture tested in table 1 was developed from several hundred viable cells and the probability that these several hundred viable cells contained any mutants appears al-

TABLE 2

THE COMPOSITION OF BACTERIAL POPULATION WITH RESPECT TO ISONIAZID RESISTANCE OF VARIOUS STRAINS OBTAINED BY ONE-STEP SELECTION OF VARIOUS ISONIAZID CONCENTRATIONS

(The Strains Were Obtained by the Experiment Shown in Table 1)

(Medium: Modified Sauton Agar Medium)

Strain*	Composition of Population† Isoniazid					
	0 γ	1 γ	3 γ	5 γ	10 γ	20 γ
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
IN-1.0	100.0	47.8	<0.13	<0.13	<0.13	<0.13
IN-2.0	100.0	124.	6.98	3.86	2.32	/
IN-3.0	100.0	106.	97.3	61.6	69.8	/
IN-5.0	100.0	64.	/	67.7	43.6	/
IN-10.0	100.0	118.	/	74.2	36.4	11.2
IN-20.0	100.0	112.	/	64.0	48.0	12.6
IN-50.0	100.0	98.	/	59.8	40.6	16.9

* Strains IN-1.0, IN-2.0, IN-3.0, IN-5.0, IN-10.0, IN-20.0, IN-50.0 represent strains (groups of approximately one hundred colonies) obtained by one-step selection of 1.0 γ, 2.0 γ, 3.0 γ, 5.0 γ, 10.0 γ, 20.0 γ, and 50.0 γ of isoniazid, respectively.

† The composition of the population is expressed as percentages of survivors on the Sauton agar medium containing various concentrations of isoniazid in the total viable bacterial population.

TABLE 3

A COMPARISON OF THE COMPOSITION OF THE POPULATION EXPRESSED AS RATIOS OF MUTANTS RESISTANT TO 10 γ OF ISONIAZID IN THE TOTAL VIABLE BACTERIAL POPULATION BETWEEN A ONE-STEP MUTANT STRAIN OBTAINED BY ONE-STEP SELECTION OF 50.0 γ OF ISONIAZID AND A MULTIPLE-STEP MUTANT STRAIN OBTAINED BY 10 TRANSFERS ONTO MEDIA CONTAINING 50.0 γ OF ISONIAZID

Strain	Number of Survivors*	
	Medium with Isoniazid 0 γ	Medium with Isoniazid 10 γ
One-step Mutant strain	184.2 (144.4–224.0) (100.0%) ($C_{\bar{x}}$ = 7.80%)	74.4 (32.0–116.8) (40.5%) ($C_{\bar{x}}$ = 20.3%)
Multiple-step Mutant strain	191.1 (166.1–216.1) (100.0%) ($C_{\bar{x}}$ = 5.82%)	221.7 (209.9–223.5) (116.0%) ($C_{\bar{x}}$ = 2.22%)

* Mean (95% confidence limits)

95% confidence limits = (mean) ± (standard error) × ("t")

$$C_{\bar{x}} = (\text{standard error}) \times (100\%) \times \frac{1}{(\text{mean})}$$

most negligible. Therefore, it would be considered that variants resistant to more than 3.0 γ of isoniazid are resistant mutants derived from spontaneous mutation, for the variants are considered to be derived from drug-susceptible cells and they are very rare. The results indicate also that the appearance rate of survivors on medium containing 3.0 to 20.0 γ of the drug are not too much different from each other. This means that most

variants resistant to 3.0 γ of isoniazid are simultaneously resistant to 20.0 γ of the drug and a number of them to 50.0 γ of the drug. Therefore, most variants resistant to 3.0 to 20.0 γ of isoniazid must be the same ones.

Accordingly, it appears that there are two groups of cells: survivors on medium containing less than 2.0 γ of isoniazid and survivors on medium containing more than 3.0 γ of isoniazid. The former are most common and the latter are rare. It appears that the isoniazid susceptibility (or resistance) of the population is not continuous at approximately 3.0 γ of isoniazid. The variants resistant to more than 3.0 γ of isoniazid must be resistant mutants.

The results obtained in *Experiments 1* and *2* (tables 1 and 2) suggest that mutants resistant to 3.0 to 50.0 γ of isoniazid, i.e., all isoniazid-resistant mutants, may be produced by a mutation of the same genotype, and isoniazid resistance may be determined by only one genotype. The results shown in tables 1 and 2 are reasonably interpreted by this assumption except for the following points.

Among the results shown in table 2, it seems that the percentage of mutants resistant to 20.0 γ of isoniazid is much lower than that of mutants resistant to 5.0 to 10.0 γ of the drug. The existence of another genotype might be suggested for the mutants resistant to more than 20.0 γ of the drug. This supposition, however, cannot interpret the similar composition of the population in the strains obtained by one-step selection of 3.0 to 50.0 γ. Therefore, it appears reasonable to assume that the same genotype exists for the mutants resistant to 3.0 to 50.0 γ of the drug. The decrease of percentages of the mutants resistant to 20.0 γ in the strains obtained by one-step selection could be interpreted as derived from the fluctuation of phenotype in the existence of the same genotype. A fluctuation of isoniazid susceptibility between 2.0 and 0 γ of isoniazid appears also to be interpreted as the fluctuation of phenotype in the existence of the isoniazid-susceptible genotype.

The results of *Experiment 3* (table 3) indicate that the character of the one-step mutant

TABLE 4*A*

RATIO OF THE NUMBER OF SURVIVORS ON MEDIA CONTAINING 3.0 γ OF ISONIAZID IN THE TOTAL VIABLE BACTERIAL POPULATION

Experiment Number	Ratio of Survivors
A	2.25×10^{-4}
B	3.46×10^{-4}
C	0.178

TABLE 4*B*

THE COMPOSITION OF BACTERIAL POPULATION OF THE STRAINS OBTAINED BY THE ABOVE EXPERIMENTS

Experiment Number	Composition of Population*			
	Isoniazid 0 γ	Isoniazid 3 γ	Isoniazid 5 γ	Isoniazid 10 γ
	per cent	*per cent*	*per cent*	*per cent*
A	100.0	97.3	61.6	69.8
B	100.0	104.	69.1	69.1
C	100.0	/	6.95	3.21

* *See* table 2.

strain is different from that of the multiple-step mutant strain. Isoniazid-resistant mutants of the former did not reproduce resistant cells of the same degree of isoniazid resistance as themselves and, therefore, they produced a heterogeneous population with respect to isoniazid resistance. Accordingly, it appears that the one-step mutant strain has a relatively unstable genotype. On the other hand, the multiple-step mutant strain represented a homogeneous population. Accordingly, it appears that the strain has a stable genotype. Mutants bearing the stable genotype would have appeared by depressing those bearing the unstable one through multiple-step selection. Thus, it has been suggested that there are two genotypes of isoniazid resistance, the one an unstable one and the other a stable one. As described above, the behavior of this organism against isoniazid is discontinuous with approximately 3 γ of isoniazid per ml. When this is true, it would be expected that the number of survivors on medium containing 3.0 γ of isoniazid expressed as percentages of the total viable population can be affected by the fluctuation of experimental conditions. This expectation has occurred as shown in table 4. The composition of the population of the strains obtained by these experiments is dependent upon the appearance rate of survivors.

In summary: It has been suggested that isoniazid susceptibility of a test strain of *Mycobacterium avium* is discontinuous with isoniazid concentrations of approximately 3 γ per ml. Bacterial cells capable of growing on 2 γ but not on 3 γ of the drug bear the isoniazid-susceptible genotype and those capable of growing in isoniazid concentrations higher than 3 γ of the drug bear the isoniazid-resistant genotype. The capability of growing on medium containing 3 to 50 γ of isoniazid per ml. appears to be determined by the same genotype. It has also been suggested that two types of the resistant genotype exist, the one an unstable one producing a heterogeneous population structure and the other a stable one producing a homogeneous population structure.

Acknowledgment

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THE RING METHOD

A Method for Analyzing the Effect of Serum on the Growth of Tubercle Bacilli *in Vitro*

In order to determine the significance of the "humoral factor" in the native and acquired resistance to tuberculosis, the present writers performed several *in vivo* experiments by using the so-called "chamber method." With this technique some new findings have been reported[1] concerning the bacteriostatic or bacteria-promoting effects of the body fluids of various animal species on various strains of mycobacteria. However, it may be more scientific to make sure of these data *in vivo* by using *in vitro* experiments.

Ordinary methods of culture are inadequate for this purpose, because body fluids, such as serum or lymph, may change their characteristics during cultivation outside the animal body and participate in reactions different from those which take place within the body. A special experimental method must be devised.

The ring method, here described, represents an attempt along this line, and it is believed at present that this method may be fairly satisfactory.

The principle of this method is contained in the following two points:

(*1*) Culture medium containing serum is replaced every two days, and thus comparatively fresh serum can continuously act on tubercle bacilli; and (*2*) the culture media are made in two different compositions with the same serum; serum is diluted with physiologic saline solution, on the one hand, and with Kirchner's liquid, on the other. Thus, the growth-promoting action of used serum on tubercle bacilli in an incomplete medium is observed by using the former serum medium; and the growth-inhibiting action of used serum in a complete medium is observed by using the latter serum medium.

The experimental procedure has already been described in the previous report,[1] but is presented here in more detail.

Preparations:

A metal ring plated with nickel (inner diameter, 1.4 cm.; thickness of ring, 0.1 cm.; height of ring, 0.35 cm.)

A smaller metal ring plated with nickel (inner diameter, 1.1 cm.; thickness of ring, 0.1 cm.; height of ring, 0.15 cm.)

Glass slides, 7.5 by 2.5 cm.

Cover-glass (3.0 by 2.5 cm.) with a small hole (0.15 cm. diameter) drilled near the center. This hole is used for replacing the culture medium.

Techniques: The smaller ring is fixed with paraffin (melting point: 72°C.) on a glass slide. A loopful of bacilli suspended in benzine is smeared on the glass slide inside the ring. After the benzine dries, the larger ring is placed so that the smaller ring is almost in the center of the larger ring (figures 1–3). The preparation is covered and the cover-glasses on the ring are made airtight with paraffin. The culture medium is poured through the hole into the inside of the smaller ring with a syringe and the space between the rings remains air-filled. The hole is then closed with paraffin. The culture medium inside the small ring does not flow out because of capillary action. The rings are placed in a Petri dish and cultured in an oven at 37°C. Forty-eight hours later a sterile injection needle is inserted into the hole and the contents are withdrawn with a syringe and new culture medium is inserted. If these procedures are done very gently, the flushing off of bacilli during the change of medium will be prevented, because the bacilli have been fixed fairly tightly on the glass surface by the benzine method. This replacing is done four to five times every two days. Ten to twelve days after the beginning of cultivation the glass slide is fixed with 10 per cent formalin and the slide is warmed with a gas flame until the paraffin becomes soft but not fluid, and the paraffin is carefully wiped off with gauze. The preparation is then stained with the Ziehl-Neelsen technique.

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[1] Tsuji, S., *et al.*: Am. Rev. Tubere., 1957, *76*, 90.

It is important to use paraffin having a high melting point, because the fixation of the rings with the glasses may come loose when paraffin with a low melting point has been used. If all manipulations are carried out under careful sterilization, there is almost no contamination even after a relatively short experience.

Culture media: Rabbits were used in the following experiments. The protein concentration of the rabbit serum varied from 7–8 per cent to 4.5 per cent, according to the condition of the animals used. Preliminarily, the relationship between the serum protein concentration and the growth of tubercle bacilli was examined and it was found that serum diluted to 3 to 5 per cent protein concentration with physiologic saline solution was always the most convenient for the cultivation of the H37Rv strain. When Kirchner's liquid medium was used in place of physiologic saline solution, the growth of tubercle bacilli improved. Consequently, serum diluted to 3 per cent protein concentration (determined by the refractometer) was used with physiologic saline solution or Kirchner's liquid.

Approximately 3 ml. of blood are collected from one rabbit by heart or vein puncture, and serum is separated by centrifugation (2,000 r.p.m. for 15 minutes). One half of the serum is diluted with physiologic saline and the other half with Kirchner's liquid so that the protein concentration of diluted serum becomes 3.0 to 3.5 per cent, respectively (in most cases these are 50 per cent dilutions of parent serum). As the amount of culture medium needed for one specimen is about 0.5 ml., 3 ml. of blood is enough for four specimens.

Preliminarily, exchanges of medium were carried out every twelve, twenty-four, and forty-eight hours with the same serum, and multiplications of bacilli were compared. The results were almost uniform under these three conditions. Therefore, the forty-eight-hour system was chosen for the subsequent experiments.

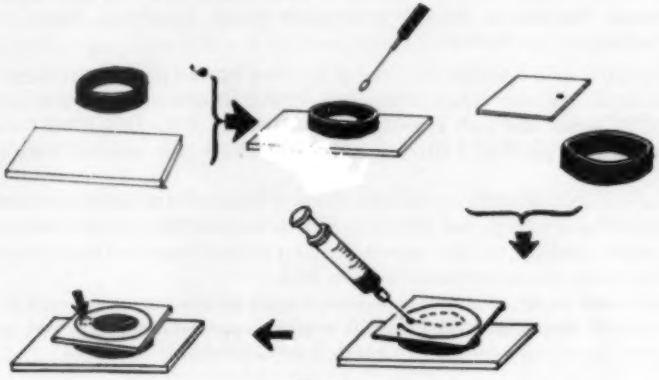

FIG. 1.

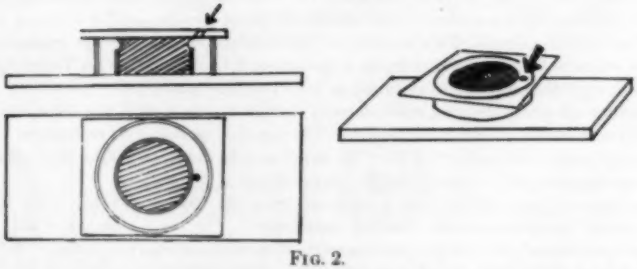

FIG. 2.

FIGS. 1 and 2. Diagrams of the technique of the "ring method."

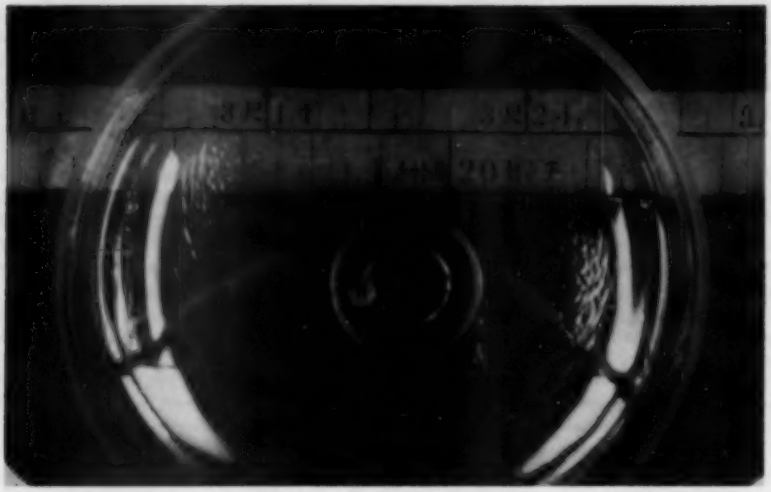

FIG. 3. Photograph of the "ring method."

All cultures were carried out with duplicate specimens. Usually the results coincided well in the duplicate specimens.

Demonstration of the tuberculostatic effect of the serum collected from the immunized rabbit: Experiments dealing with immunization with subcutaneous inoculation of 1 mg. of the living H37Rv strain and with intramuscular inoculation of the heat-killed bovine RM strain suspended in paraffin oil (100 mg. and 200 mg.) have been reported in the previous paper.[1]

In these cases, apparent tuberculostatic effects of immunized serum were demonstrated in the serum diluted with physiologic saline; and, on the other hand, in the medium diluted with Kirchner's medium, bacillary growth was good in both immunized and normal control systems and there was no difference between them.

In the present paper, additional experiments using rabbits immunized with the living BCG strain will be described. Male rabbits weighing approximately 3 kg. were used, and the negative tuberculin responses of all animals were previously confirmed.

Eight rabbits were divided into four groups; there were 2 animals in each group. Inocula of 1 mg., 5 mg., and 50 mg. of BCG suspended in 1 ml. of physiologic saline solution were inoculated into the back muscles of the rabbits in three groups, and one group remained untreated as control animals. Two weeks after inoculation, the tuberculin response of each animal was examined (by intracutaneous injection of 0.1 ml. of 1:10 Old Tuberculin), and all animals except the controls were found to give positive reactions.

Experiments adopting the ring method were started on the eighth day after the inoculation of BCG and ended on the twentieth day. During that period four exchanges of culture medium containing serum collected from the same animal every two days were made. (This series of experiments will be called the first experiment.)

Another series of experiments was performed from the thirty-first day until the forty-third day, using the same animals (second experiment). The body weights of animals used were almost unchanged, and the protein concentration of blood also remained within the normal range during the experimental period in spite of frequent withdrawals of blood.

Tubercle bacilli used in the culture were from the H37Rv strain that had been cultured for three weeks on Sauton's medium.

The results are summarized in table 1. In the first experiment, the immunizing effects of inoculated BCG might have yet been insufficient because of the relatively short period after the immunization. Nevertheless, the bacillary growths in the serum-saline culture medium from the animals inoculated with 5 mg. and 50 mg. of BCG were apparently inhibited when compared with the growth in the control specimens. In the serum-Kirchner culture medium the bacillary growths were vigorous, and there was no difference between the immunized and control specimens.

In the second experiment, there was no growth at all of the tubercle bacilli in the serum-saline medium made of serum from the rabbit immunized with 50 mg. of BCG. In the serum-saline medium made of serum from the animal immunized with 5 mg. of BCG, apparent restriction of bacillary growth was also confirmed. No difference in bacillary growth was observed, however, between the serum-saline medium of the rabbit immunized with 1 mg. of BCG and the control serum.

In the second experiment, unlike the first experiment, bacillary growths were slightly depressed in the serum-Kirchner medium of immunized sera (with 5 mg. and 50 mg. of BCG). Serum of the animal immunized with 1 mg. of BCG did not differ from control serum in both the first and second experiments.

* * *

The results described above indicate that the immune serum has a tuberculostatic effect on virulent human tubercle bacilli. From the culture data with serum-saline medium, it is inferred that one of the causes of this bacteriostatic effect of immune serum may be the decrease of the growth-promoting effect which is possessed by the normal serum. The growth-inhibitory effect, on the other hand, is assumed to increase, although slightly, in the immune serum dealt with in the second experiment (depression of growth in the serum-Kirchner medium). This is slightly different from the data described in the previous report.

In conclusion, from the experimental results reported in the previous paper and the results obtained here, it seems definite that a tuberculostatic effect does develop in the

TABLE 1

BACILLARY GROWTH OF H37Rv STRAIN CULTURED BY THE "RING METHOD" IN THE SERUM COLLECTED FROM THE RABBIT IMMUNIZED WITH LIVING BCG VACCINE

BCG Inoculation	First Experiment		Second Experiment	
	Diluted with Kirchner Medium	Diluted with Saline	Diluted with Kirchner Medium	Diluted with Saline
mg				
1	+++	++	+++	++
5	+++	+	++	+
50	+++	+	++	−
Control	+++	++	+++	++

+++ Colonies adhere to each other and no distinct colonies are seen.
++ Some colonies adhere to each other, but many distinct colonies are seen.
+ Very small distinct colonies.
− No bacillary growth.

serum of the rabbit sufficiently immunized with living or dead tubercle bacilli. It further appears that the main cause of this effect is the reduction of the growth-promoting effect which is contained in the serum of the normal nonimmunized rabbit. However, the possibility of the increase of growth-inhibiting action, also, cannot be excluded as a possible consequence of immunization.

In summary: A new slide culture method for studying tubercle bacilli in the serum medium has been described in detail. This is called the "ring method." There are two principal characteristics of this method: (*1*) Culture medium containing serum is replaced every two days and thus comparatively fresh serum is capable of acting continuously on tubercle bacilli, and (*2*) the growth-promoting action of the used serum on tubercle bacilli (in an incomplete medium diluted with physiologic saline solution) and the growth-inhibitory action of the same serum (in a complete medium diluted with Kirchner's liquid) can be determined separately. By using this method, the development of the tuberculostatic effect in the serum of the immunized rabbit has been demonstrated. It appears that the development of this tuberculostatic effect in the immunized animal may be brought about mainly by the reduction of the growth-promoting effect contained in the normal nonimmunized serum.

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August 20, 1957

THE POSSIBILE ROLE OF HUMORAL FACTORS IN THE ENHANCED GROWTH OF TUBERCLE BACILLI IN RABBITS GIVEN CORTISONE

At present it is generally recognized that tuberculosis in man is frequently influenced deleteriously by the administration of pharmacologic doses of cortisone or corticotropin.[1] In tuberculosis experimentally induced in animals, very conflicting results have been reported by investigators.[2] It is certain, however, that under certain experimental conditions cortisone can act deleteriously on animal tuberculosis.

The fundamental mechanism of this deleterious effect of cortisone has not yet been entirely clarified. Some investigators[3-5] ascribed this deleterious effect on animal infection to the depression of antibody formation and the inhibition of antigen-antibody reaction by hormone. Other investigators[6-8] ascribed the reduction of resistance to tuberculosis by hormones mainly to the alteration of functions of phagocytes, and Lurie,[9] especially, has strongly denied the role of humoral factors. Robson and associate,[10] who studied the influence of cortisone by adopting the experimental method of direct observation of intracorneal tuberculosis in mice, found a striking inhibition of cellular invasion in connection with a marked enhanced multiplication of innoculated extracellular tubercle bacilli. They suggested, although they did not discuss the possible role of the humoral factors, that this enhanced growth of extracellular bacilli had been brought about by the prevention of phagocytosis by hormones.

Thus, it appears that no one has been able to demonstrate clearly that there was increased growth of tubercle bacilli in the animal treated with cortisone, and that this stimulating action of hormones on the growth of tubercle bacilli was related, at least partially, to the alteration of the innate defensive power of the body fluid of the animal. Even if the role of the altered function of phagocytes may be accepted, the role of humoral factors must be further investigated.

The present writers[11] investigated the role of humoral factors in native and acquired resistance to tuberculosis, and reported various interesting results obtained by "the chamber method" *in vivo* and the results obtained by the "ring method" *in vitro*. From these results it is believed that humoral factors play a significant role in resistance to tuberculosis.

In the present paper it will be demonstrated that a certain change of humoral factors may induce the stimulated multiplication of tubercle bacilli in the cortisone-treated animal *in vivo* and that the serum collected from this animal has a stronger growth-promoting action on tubercle bacilli than the serum of the nontreated control animal.

Male rabbits weighing 2.5 to 3.0 kg. were used in these experiments. Cortisone acetate was used and the dosage, duration, and period of administration of this hormone will be noted in each section.

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[1] American Trudeau Society: Am. Rev. Tubere., 1952, *66*, 254.
[2] American Trudeau Society: Am. Rev. Tubere., 1952, *66*, 257.
[3] Gemuth, F. G., Jr., and Ottinger, B.: Proc. Soc. Exper. Biol. & Med., 1950, *74*, 851.
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[5] Solotorovsky, M., *et al.*: Proc. Soc. Exper. Biol. & Med., 1951, *76*, 286.
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[7] Lurie, M. B.: Advanc. in Tubere. Research, 1955, *6*, 18.
[8] Youmans, G. P., and Youmans, A. S.: Am. Rev. Tubere., 1954, *69*, 790.
[9] Lurie, M. B., *et al.*: Science, 1951, *113*, 234.
[10] Robson, J. M., and Didcock, K. A.: Am. Rev. Tubere., 1956, *74*, 1.
[11] Tsuji, S., *et al.*: Am. Rev. Tubere., 1957, *76*, 90.

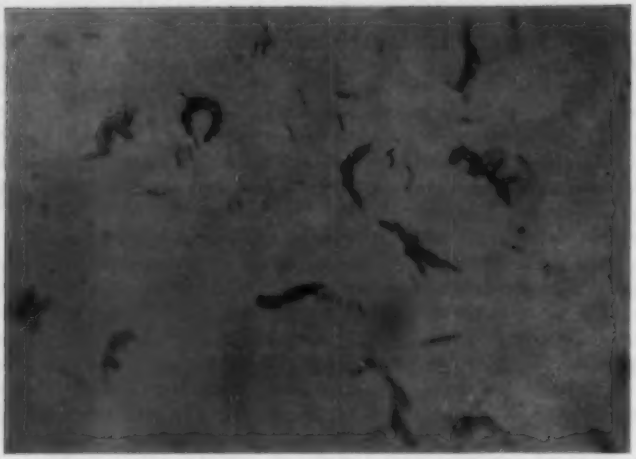

FIG. 1. Growth of H37Rv strain in the *O*-chamber of untreated control animal.

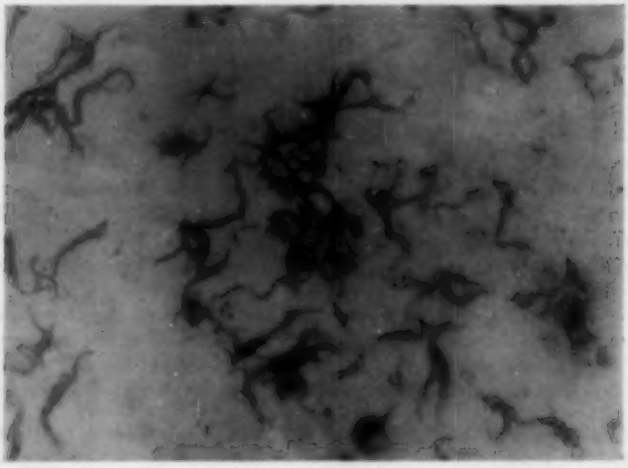

FIG. 2. Growth of H37Rv strain in the *O*-chamber of cortisone-treated animal.

Body weights of experimental animals were measured every day, and the number of lymphocytes in circulating blood was counted every other week. All rabbits treated with cortisone showed a marked decrease of lymphocytes after one week of treatment. The loss of body weight was frequently marked after three weeks of hormone administration.

The chamber method has already been reported in detail[12] and, therefore, the description will be omitted here. By this method one can observe the growth of tubercle bacilli in the body fluid completely separated from cells *in vivo* (*O*-chamber) and in a medium in which only the lower molecular fraction of body fluid can act on tubercle bacilli *in vivo* (the *K*-chamber).

[12] TSUJI, S., AND ITO, K.: Am. Rev. Tuberc., 1955, *72*, 393.

Five chambers smeared with H37Rv, three of which were *O*-chambers and the other two *K*-chambers, were implanted intraperitoneally into one rabbit. Seven rabbits were treated intramuscularly with 5 mg. per kg. of cortisone acetate every day from the day of implantation. Six rabbits were untreated controls. Two weeks after the implantation, all rabbits including controls were sacrificed by heart puncture and all of the artificial chambers were taken out.

Bacillary growth in the chambers was examined by low-power magnification and by oil-immersion lens, and sizes and forms of colonies were compared. Bacillary growth in the

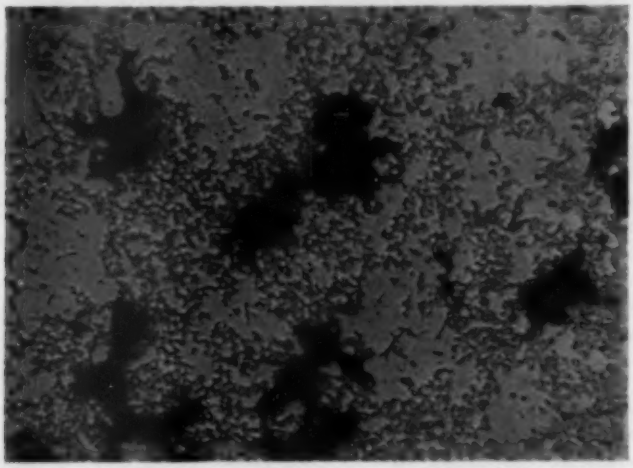

FIG. 3. Growth of H37Rv strain in the normal serum diluted with Kirchner's medium. Some colonies adhere to each other, but many distinct colonies are seen.

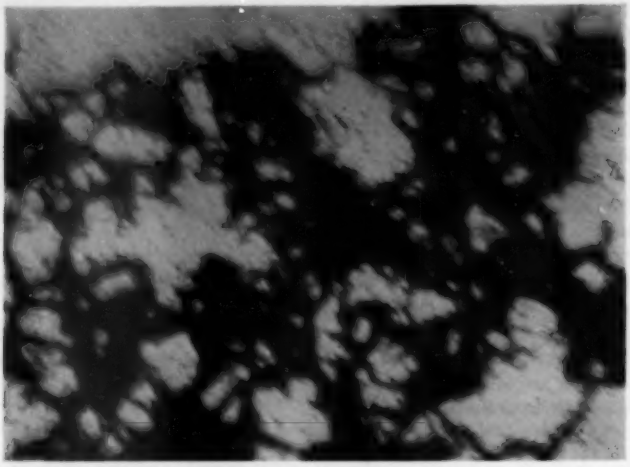

FIG. 4. Growth of H37Rv strain in the cortisone serum diluted with Kirchner's medium. Colonies adhere to each other, and no distinct colonies are seen.

respective *O*-chambers in the control animals was not always uniform, owing, presumably, to the individual differences in native resistance of the rabbits used. These differences, however, were very slight and bacillary growth in the chambers from the same animals was of the same degree. Generally, the colonies in the control chambers could be barely distinguished by low-power magnification, and took the form of a point or a very short "stick;" typical serpentine forms were very rare (figure 1). On the other hand, colonies demonstrated in the *O*-chambers from the cortisone-treated rabbits were easily recognized by low-power magnification, and the forms of these colonies were almost always typical serpentine forms and many colonies adhered to one another (figure 2). Differences in sizes and forms of colonies between the control and the cortisone-treated *O*-chambers were very conspicuous.

In another experiment in which duration of cultivation was shortened to only one week, the superiority of the growth of bacilli in the cortisone-treated chambers was confirmed.

Similar experiments were performed by using the bovine RM strain of *M. tuberculosis*, and results similar to those described above were obtained.

No bacillary growth was found in the *K*-chambers implanted into both cortisone-treated and nontreated animals.

* * *

From these experimental results it was concluded that the virulent tubercle bacilli were markedly stimulated in their extracellular growth *in vivo* by the daily administration of cortisone acetate to the host for two weeks.

As the direct action of phagocytes on bacilli is eliminated in this experiment, it may be assumed that the body fluid of an animal treated with a pharmacologic dose of cortisone has altered its character so that the growth of tubercle bacilli is stimulated extracellularly.

It is rather surprising that Lurie found, on the contrary, that the growth of tubercle bacilli was markedly suppressed when they were cultured in the collodion-coated silk bag *in vivo*. This method is very complicated, however, and perhaps unhomogeneous distribution of bacilli in the silk bag may have endangered the certainty of the results obtained by the quantitative cultivation of agar pieces. The present findings obtained by the *O*-chamber appeared quite definite.

In addition it is very interesting and noteworthy that Robson's finding that tubercle bacilli are markedly stimulated in their extracellular growth in the cornea of the mouse treated with cortisone agrees very well, as a matter of fact, with the findings obtained by the chamber method.

Naturally, the anti-inflammatory action of cortisone must be considered. The exudate around the chamber, as Lurie[13] pointed out, may be different in amount and probably in quality also between the animal under the influence of cortisone and the animal not under such influence. Consequently, it is reasonable to believe that the enhanced growth of tubercle bacilli in the *O*-chamber may be attributed to the alteration, brought about by decreased cell reaction, in the character of the liquid which diffuses into the chamber and not to the primary change in character of body fluid due to hormones.

A more definite evidence of the influence of cortisone administration on body fluid would be found, however, if it could be demonstrated *in vitro* that the action of the body fluid of cortisone-treated animals is more growth stimulating for tubercle bacilli than that of the body fluid of untreated control animals.

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[13] LURIE, M. B.: Personal communication.

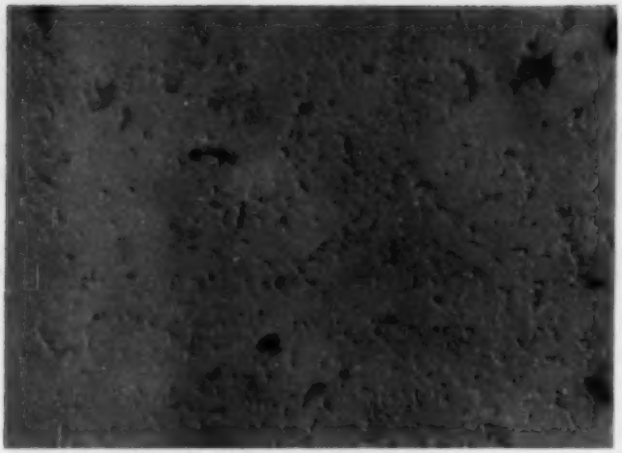

FIG. 5. Growth of H37Rv strain in the normal serum diluted with physiologic saline. Very small distinct colonies.

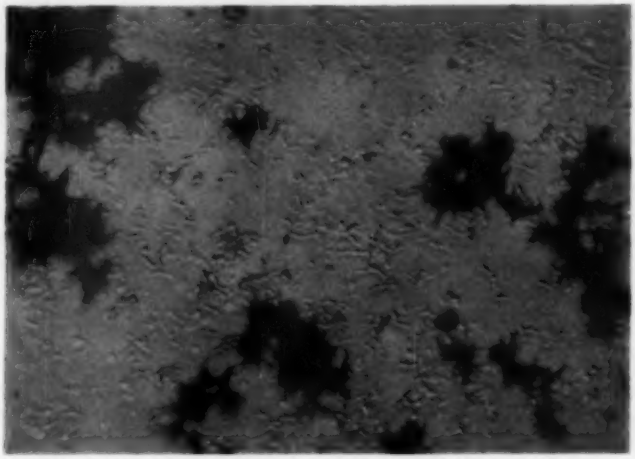

FIG. 6. Growth of H37Rv strain in the cortisone serum diluted with physiologic saline. Some colonies adhere to each other, but many distinct colonies are seen.

Accordingly, *in vitro* experiments using the "ring method" were performed. This method has already been reported and the principle of the method is described in a paper[14] which immediately precedes this report.

Three rabbits were treated with cortisone in a 5 mg. per kg. dosage every day for forty-two days. On the seventh day, 3 ml. of blood were collected from each animal, and the

[14] TSUJI, S., *et al.*: Am. Rev. Tubere., 1958, 77, 524.

TABLE 1

SUMMARIZED RESULTS OBTAINED BY THE RING METHOD

First Experiment				Second Experiment			
Diluted with Kirchner Medium		Diluted with Saline		Diluted with Kirchner Medium		Diluted with Saline	
Cortisone Serum	Control Serum	Cortisone Serum	Control Serum	Cortisone Serum	Control Serum	Cortisone Serum	Control Serum
+++	++	++	+	+++	++	+	+
Inhibitory power <		Promoting power >		Inhibitory power <		Promoting power =	

+++ Colonies adhere to each other and no distinct colonies are seen.
++ Some colonies adhere to each other, but many distinct colonies are seen.
+ Very small distinct colonies.

experiment using the ring method with the separated serum of this blood was started. After that, on the ninth, eleventh, thirteenth, fifteenth, and seventeenth days, exchanges of serum medium with fresh serum collected from the same animal were made. Cultivation ended on the nineteenth day (first experiment).

From the thirtieth day, similar cultivation was started again and ended on the forty-second day (second experiment).

Bacillary growth on the slides was compared with that of untreated controls in both experiments.

Typical examples of these specimens are shown in figures 3, 4, 5, and 6. In the first experiment the difference between the cortisone serum and the normal serum was very conspicuous. Bacillary colonies cultivated in the cortisone serum were considerably larger than those cultivated in the normal serum. This difference was confirmed in both the medium diluted with Kirchner's liquid and the medium diluted with physiologic saline.

In the second experiment the results were somewhat indefinite. Bacillary growth in the cortisone serum diluted with Krichner's medium was decidedly superior to that in the normal serum diluted with the same medium, but the difference was not apparent in the medium diluted with saline. The results are summarized in table 1.

* * *

In the first experiment the tuberculo-inhibitory power was reduced and the tuberculo-promoting power increased in the serum treated with cortisone for three weeks. On the other hand, after four weeks' administration these conditions changed, and though the tuberculo-inhibitory power was still weaker in the cortisone serum, the tuberculo-promoting action was reduced to the degree of normal serum.

It is evident from these results that the serum collected from the animal under the influence of cortisone enhances the multiplication of tubercle bacilli *in vitro*. This enhancing effect of serum becomes weaker, however, when cortisone administration is continued for more than four weeks, owing to the reduction of the growth-promoting function of serum to the normal degree. And when serum becomes opalescent after too strong an influence of cortisone, the serum acquires a rather tuberculostatic effect. (This has been proved by the slide culture method[15]).

In summary: In order to determine the significance of the humoral factor in the in-

[15] Unpublished data.

creased growth of tubercle bacilli in the cortisone-treated animal, some experiments were performed *in vitro* and also *in vivo* by the chamber technique. It was demonstrated that virulent tubercle bacilli (the H37Rv strain and the bovine RM strain) grew apparently more rapidly and more vigorously in the body fluid completely separated from cells in the cortisone-treated rabbit *in vivo* than in the body fluid from the normal untreated rabbit. It was also observed that serum collected from an animal treated with cortisone had a definite growth-stimulating action on tubercle bacilli.

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August 20, 1957

THE EFFECT OF ADRENOCORTICAL HORMONES ON EXPERIMENTAL TUBERCULOSIS IN ADRENALECTOMIZED MICE

Considerable controversy exists concerning the effect of the adrenocortical hormones on experimental tuberculosis. The use of cortisone alone has generally been accepted as having an adverse effect upon tuberculosis in experimental animals and man.[1] In mice, cortisone has been shown to increase the susceptibility of mice to tuberculosis[2] and to stimulate the multiplication of the bacilli and the severity of the disease in intact[3, 4] and in adrenalectomized animals.[5] It has also been reported that adrenalectomy produces a slightly favorable effect on the course of the disease.[5]

The purpose of the present study is to investigate the effect of other adrenocortical hormones in various concentrations on the severity of experimental tuberculosis in mice

Initially 20 intact and 20 bilaterally adrenalectomized CF1 albino mice were infected with *M. tuberculosis* var. *hominis* H37Rv by means of an air-borne infection apparatus.[6] The mice were infected four days after adrenalectomy. The adrenalectomized mice were maintained on a drinking solution of 0.9 per cent sodium chloride throughout the experiment. The mice were examined at autopsy at three and one-half and seven weeks after infection and the lungs were examined grossly. Pieces of the lungs were fixed in 10 per cent formalin. For histologic examination, sections were stained with Harris' hematoxylin and eosin stain and with Ziehl-Neelsen acid-alcohol stain.

A second group of 230 mice were adrenalectomized and, along with 35 intact mice, were infected as before with *M. tuberculosis*. The adrenalectomized mice were divided into seven groups: (*1*) oil-treated controls; (*2*) animals which received 50 γ of hydrocortisone[7] per animal per day; (*3*) animals which received 500 γ of hydrocortisone per animal per day; (*4*) animals which received 50 γ of desoxycorticosterone[7] per animal per day; (*5*) animals which received 500 γ of desoxycorticosterone per animal per day; (*6*) animals which received 50 γ of hydrocortisone and 50 γ of desoxycorticosterone per animal per day; (*7*) animals which received 10 γ of 2-methyl-9-fluorohydrocortisone[7] per animal per day.

The daily dose in each case was administered in 0.1 ml. of corn oil. The intact infected animals were treated only with oil. Approximately half of the animals were sacrificed at two and one-half weeks and the remainder at five weeks after the infection. The lungs were examined grossly and histologically. In addition, one lobe of each lung was placed with others from the same group in 12½ per cent trisodium phosphate and the combined lobes were homogenized with a Virtis® homogenizer. The homogenates were centrifuged at 1,200 *g*. for fifteen minutes, and smears were made of the concentrates. The remainder of the concentrates were diluted with Sorenson's buffer, pH 6.6, and various concentrations were added in duplicate to plates containing RDM-1 medium.[8] The plates were incubated at 37° C. and the number of colonies of bacilli were counted after three- and four-week incubation periods.

In the first series of animals, the adrenalectomized mice showed a slightly greater in

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[1] Johnson, J. R., and Davey, W. N.: Am. Rev. Tuberc., 1954, *70*, 623.

[2] Hart, P. D'A., and Rees, J. W.: Lancet, 1950, *2*, 391.

[3] Ilavsky, J., and Foley, E. J.: Antibiotics, 1954, *4*, 1068.

[4] Robson, J. M., and Didcock, K. A.: Am. Rev. Tuberc., 1956, *74*, 1.

[5] Naguib, M., and Robson, J. M.: Brit. J. Pharmacol., 1955, *10*, 387.

[6] Middlebrook, G.: Proc. Soc. Exper. Biol. & Med., 1952, *80*, 105.

[7] The hormones were generously donated by Dr. Robert O. Stafford, Department of Endocrinology, Upjohn Co., Kalamazoo, Michigan.

[8] Taylor, R. L., *et al.*: Laboratory Methods for Tuberculosis Bacteriology, Report #1 revised, U. S. Army Research and Development Unit, Fitzsimons Army Hospital, Denver, Colorado, 1957.

TABLE 1

A SUMMARY OF THE INFECTION DATA ON ADRENALECTOMIZED MICE WITH VARIOUS HORMONE TREATMENTS

The Relative Severity of the Disease is Estimated by the Means of Varying Numbers of (+)'s

	Mortality	Smears	Cultures	Gross Lung Lesions	Histologic Examination
		Examined for ercle TubBacilli			
	per cent				
Infected animals					
Intact controls (*35*)*	3	+	+++	++	++
Adrenalectomized controls (*40*)	58	+	+	+++	+++
Adrenalectomized + 50 γ hydrocortisone (*35*)	3	++	+++	+++	+++
Adrenalectomized + 500 γ hydrocortisone (*35*)	6	++++	+++++	+++	+++++
Adrenalectomized + 50 γ desoxycorticosterone (*35*)	28	+	++	++	++
Adrenalectomized + 500 γ desoxycorticosterone (*40*)	22	++	+++	++	++++
Adrenalectomized + 50 γ hydrocortisone + 50 γ desoxycorticosterone (*31*)	19	+	+	++	+++
Adrenalectomized + 10 γ 2-methyl-9-fluorohydrocortisone (*15*)	53	++	++++	+++	++++
Noninfected animals					
Adrenalectomized controls (*34*)	88	−	−	−	−

* Total number of mice in each group

Criteria for grading severity of infection

Smears:
+ = a few isolated bacilli
++ = a fair number of bacilli
++++ = smear loaded with bacilli

Cultures (number of colonies of bacilli):
+ = 4,000–10,000
++ = 10,000–25,000
+++ = 25,000–100,000
++++ = 100,000–300,000
+++++ = 300,000–1,000,000

Gross lung lesions (average):
+ = a few isolated lesions
++ = approximately ¼ of lung surface with lesions
+++ = approximately ½ of lung involved
++++ = almost entire lung covered with lesions

Histologic observations: The degree of infection in the lungs of the intact controls was arbitrarily classified as (++). The experimental groups were compared with the controls and graded with varying numbers of (+)'s depending on amount of lung involvement and degree of tissue reaction.

fection than the intact mice, both grossly and histologically, after three and one-half weeks of infection. The lungs of animals from both groups demonstrated discrete lesions, a greater number being observed in the lungs of the operated animals. After seven weeks of infection, both groups demonstrated very severe disease and no differences could be observed.

A summary of the observations on the second group of mice is found in table 1. The mortality in both adrenalectomized, untreated groups was high, but the mortality in the uninfected animals was higher than in the infected. It does not seem probable that infection would increase the survival rate of adrenalectomized animals, but at present this seems to be the case, the infection being the only difference in the way the two groups of animals were handled. Generally, the animals receiving the high dose of hydrocortisone demonstrated the greatest effect of the disease. After two and one-half weeks of infection, the intact and adrenalectomized mice showed signs of a very early stage of the disease, each having lungs with a few small, discrete lesions. All of the hormone-treated animals showed more disease than the controls, and the lesions were more diffuse.

Five weeks after the infection, the lungs of the adrenalectomized controls contained more lesions than those of the intact animals. At low-treatment levels with hydrocortisone and desoxycorticosterone, the extent of the disease was similar to that in the untreated adrenalectomized group, but the lesions were more diffuse in character. The high level of hydrocortisone caused a marked increase in the extent of the disease and was associated with diffuse lesions. The high dosage of desoxycorticosterone caused an increase in the disease only slightly less than hydrocortisone and the lesions were diffuse. The 2-methyl-9-fluorohydrocortisone demonstrated an infection almost as intense as that seen with the high levels of the other hormones, but the lung lesions were more discrete.

All of the adrenalectomized animals were maintained on 0.9 per cent sodium chloride as drinking water to keep the experimental conditions constant, despite the mineralocorticoid activity of desoxycorticosterone and 2-methyl-9-fluorohydrocortisone. The daily water consumption of each experimental group was recorded, and no significant differences were observed. Each of the groups gained approximately the same amount of weight during the five-week period of infection.

It would appear that under these conditions at high dose levels, desoxycorticosterone demonstrates a final effect on the animal's response to tuberculosis similar to that of hydrocortisone which has been shown to be an anti-inflammatory substance.[9] Although 2-methyl-9-fluorohydrocortisone has been reported to be many times more active than hydrocortisone as an anti-inflammatory substance,[10] its effect in this study varies from that of hydrocortisone in view of the presence of different types of lesions.

In summary: Adrenalectomy in mice appeared to decrease the animal's ability to combat experimental infections with *M. tuberculosis*. In adrenalectomized mice hydrocortisone and desoxycorticosterone, especially in high doses, caused an increase in the severity of the disease and more diffuse lesions appeared in the lungs. The compound 2-methyl-9-fluorohydrocortisone also caused an increase in the severity of the disease at low doses, but the lung lesions resembled those of the control-animals.

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[9] Menkin, V.: Science, 1956, *123*, 527.
[10] Byrnes, W. W., *et al.*: Proc. Soc. Exper. Biol. & Med., 1956, *91*, 67.
[11] Present address: Department of Biology, University of Oregon, Eugene, Oregon.

FURTHER EXPERIENCE WITH SINGLE-DRUG (ISONIAZID) THERAPY IN CHRONIC PULMONARY TUBERCULOSIS[1]

Initial Therapy with High-Dose Isoniazid

In 1954 Deuschle and associates[2] reported the results in a series of 47 cases of chronic pulmonary tuberculosis treated with isoniazid alone in doses of 5 mg. per kg. of body weight. In that study approximately two-thirds of the original patient group were classified as having shown substantial roentgenographic improvement and one-quarter were still excreting tubercle bacilli in their sputum at the end of the therapeutic year. The present group of patients represents an extension of this experience at the New York Hospital-Cornell Medical Center and the Navajo Medical Center, utilizing an isoniazid dosage schedule of 10 mg. per kg. for the initial three weeks of therapy and 5 mg. per kg. for the remainder of the treatment period.

High doses of isoniazid in the range of 10 or more mg. per kg. are associated with some risk of neurotoxicity. Pyridoxine exerts a neuritis-preventing action in man, but there is some evidence that this may be accomplished at the cost of a loss of some portion of the antimicrobial effectiveness of the isoniazid.[3, 4] The regimen employed, therefore, represented an attempt to obtain the benefits of a high dose of isoniazid during the early stages of treatment without risk of partial neutralization by pyridoxal, while at the same time minimizing neurotoxicity by the reduction in isoniazid dosage at the end of the third week.

Only patients with moderately advanced or far advanced pulmonary tuberculosis were admitted to the study group. Of 22 patients initially included at the Navajo Medical Center (Fort Defiance, Arizona), 6 were eliminated: 4 patients because of leaving against medical advice within the first three months of treatment; one because the type of disease, pleural effusion, did not meet the criteria for inclusion in the series; and one because the patient requested transfer to another institution early in the course of therapy. None of the 16 patients at New York Hospital-Cornell Medical Center begun on this treatment program were eliminated.

All of the findings in the New York cases were bacteriologically confirmed. Tubercle bacilli were not isolated from 3 of the Fort Defiance patients, but the roentgenographic findings and the character of the therapeutic response were sufficiently indicative of tuberculosis to warrant their inclusion in the series.

The tuberculosis in 10 of the 16 New York patients was classified as moderately advanced, and in 6 it was far advanced; of the combined group of 32 patients the disease was moderately advanced in 15 and far advanced in 17 patients. Cavities were present at the start of therapy in all of the New York cases and in 14 of the 16 Fort Defiance cases for a combined total of 30 from the group of 32.

None of the patients had had any previous antimicrobial therapy for their tuberculous disease.

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[1] This study was supported in part by grants from the Navajo Tribal Council, the American Trudeau Society, Medical Section of the National Tuberculosis Association (a special bequest from the Estate of Sophie Goman); Research Grant E-635, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service; and the Max C. Fleischmann Foundation of Nevada.

[2] DEUSCHLE, K., *et al.*: Am. Rev. Tuberc., 1954, *70*, 228.

[3] MCCUNE, R., *et al.*: Am. Rev. Tuberc., 1957, *76*, 1100.

[4] BOONE, I. U., *et al.*: Am. Rev. Rubere., 1957, *76*, 568.

During the therapeutic year, 21 (65 per cent) of the 32 patients completed their course without need of additional medical or surgical therapy. A slightly greater proportion of this group, 12 of 16, were Fort Defiance cases whereas 9 of 16 were New York cases. The roentgenographic extent of disease before therapy seemed to bear no significant relation to the success of the therapy.

In 11 of the 32 patients the course during isoniazid therapy was such that it was decided to employ additional therapeutic measures, i.e., surgical excisions or a change in chemotherapy. Again there was no significant difference between the roentgenographic extent of disease in this group requiring additional therapy and in the group as a whole. Of these 11 patients, 4 had persistent cavitation with infectious sputum and 5 patients had persistent cavitation with noninfectious sputum at periods varying from six to eleven months after the beginning of treatment. In the remaining 2 patients, the initial therapy was changed because of complicating factors not directly related to the tuberculous lesions.

Drug toxicity was not observed in any of the patients treated with this isoniazid regimen.

Roentgenographic course: Following three months of therapy, two-thirds of the total group had shown substantial roentgenographic improvement, and at the end of six months this had occurred in three-quarters of the group. The degree of improvement was slightly greater in the Fort Defiance cases than in the New York cases. Of the 30 patients in whom there was roentgenographic evidence of cavitation, in 19 (63 per cent) the cavities were lost to view during therapy. Ten of these 19 patients reached this point during the first three months of chemotherapy.

Bacteriologic course: As was noted above, 3 of the Fort Defiance patients never had demonstrable tubercle bacilli in the sputum. Of the remaining 29 patients, 23 (79 per cent) attained noninfectious status. In 19 of these 23 cases the disappearance of tubercle bacilli from the sputum occurred during the first three months of treatment.

Of the 29 patients who were discharging tubercle bacilli when treatment was started, 8 (27 per cent) discharged bacilli resistant *in vitro* to isoniazid concentrations of one γ per ml. on at least one occasion during the treatment period.

Of the 11 patients who required additional therapy for the management of their disease, 5 discharged tubercle bacilli which were resistant to isoniazid concentrations of at least one γ per ml. on at least one occasion. It is noteworthy that 3 of these patients excreted tubercle bacilli susceptible to isoniazid on subsequent observations.

Of the 2 patients who were considered to have roentgenographic deterioration one discharged only isoniazid-susceptible organisms. The other patient with roentgenographic deterioration, followed by clearing without change in therapy, discharged resistant bacilli on two occasions but subsequently excreted isoniazid-susceptible organisms.

Analysis of cases in which additional therapy was employed: Of the 11 cases in which additional therapy was instituted, there were 6 which were not treatment failures in the strict sense. In 4 of these patients, a considerable degree of improvement had been observed before surgical treatment was recommended. In these patients excellent results were obtained from excisional therapy with additional drugs for the surgical period in accordance with routine local practice. Two patients did very well on added chemotherapy. In 3 patients the final result was not satisfactory.

If the patients who were successfully treated with additional measures are classified as favorable results, the outcome was satisfactory in 29 (90 per cent) of the 32 patients.

Of the 2 cases with the classification of roentgenographic deterioration, one merely represented an increase in density without an increase in the size of the diseased area or

extent of cavitation. Surgery was performed at an early date in this patient, principally because of the possible presence of a complicating bronchogenic carcinoma. The other patient is the only one in the entire group in whom an unequivocal spread of disease took place while she was on isoniazid therapy. It should be emphasized that this apparently new area of disease cleared entirely without change in the single-drug isoniazid therapy. Subsequently additional drugs were incorporated in the regimen because of persistence of the initial cavitation and the continued discharge of tubercle bacilli in the sputum. This patient subsequently had a good result with surgery.

* * *

These results are distinctly more successful than those which were observed in the previously reported series, in which one-third of the patients who completed one year of single-drug therapy still excreted tubercle bacilli and only one-third of those with cavities attained cavity closure without additional therapeutic measures. The superior results, however, cannot be necessarily attributed to the higher dosage of isoniazid administered initially to the patients in the present series. All of the present patients were previously untreated whereas in the former series nearly half were "re-treatment cases" who had received other antimicrobial therapy at some time in the past. In the former series treated in the 1951–1952 period, even among the previously untreated patients there were more with long-standing chronic disease. Thus the earlier series represented a markedly less favorable group with respect to chemotherapy than the present series of patients.

A more significant comparison of results may be made with a series of patients treated in the same two institutions with pyrazinamide-isoniazid.[5, 6] This series, though not treated concurrently, is comparable as these patients were also previously untreated. The only significant selection factor favors the pyrazinamide-isoniazid series since 9 patients with minimal disease were included, whereas the present series is composed only of cases of moderately and far advanced disease. In 55 patients treated with pyrazinamide-isoniazid, for whom data were available at six months, reversal of infectiousness resulted in 93 per cent, substantial roentgenographic improvement in 76 per cent, and closure of all cavities in 58 per cent of those with cavitary lesions. In the present series the corresponding values at six months were: reversal of infectiousness in 72 per cent, substantial roentgenographic improvement in 78 per cent, and closure of cavities in 57 per cent. In both series there were only slight further gains in reversal of infectiousness and in cavity closure during the second six months except for those induced by surgical or other additional measures. Unequivocal roentgenographic deterioration occurred during the year in none of the pyrazinamide-isoniazid-treated and in only one of the present series of patients. Only with respect to reversal of infectiousness are the results in the two-drug pyrazinamide-isoniazid regimen undoubtedly superior to the single-drug isoniazid regimen. The superiority in this respect is offset by the contrast in toxicity between the two regimens. There was no toxicity in the present series, but in the pyrazinamide-isoniazid series there were 6 instances of hepatic toxicity and one death from this cause. Pyrazinamide-isoniazid, though no longer regarded as an acceptable regimen because of this factor of toxicity, is recognized as a two-drug regimen of maximal therapeutic efficacy, at least equal to any of the standard multiple-drug regimens. It can therefore serve as a significant frame of reference. Thus, in the writers' experience the single-drug isoniazid regimen has been found to be not the equal in effectiveness but in the same general class of effectiveness as pyrazinamide-isoniazid and the other multiple-drug regimens.

The results in the present series are in agreement with those of the earlier study, indi-

[5] McDermott, W., *et al.*: Am. Rev. Tubere., 1954, *69*, 319.
[6] Muschenheim, C., *et al.*: Am. Rev. Tubere., 1954, *70*, 743.

cating that *in vitro* resistance to isoniazid is less well correlated with clinical progress than is the case with the other major antituberculous agents. The implications of this singularly nontoxic and simple treatment regimen for groups who have little or no access to hospital care was previously emphasized. Others have also reported sustained clinical benefit in pulmonary tuberculosis from long-term treatment with isoniazid alone,[7, 8] and results as good as those with multiple-drug regimens in certain categories of cases.

It should be noted that in the patients of the present group in whom resectional therapy was eventually required, surgery could be undertaken with the addition of streptomycin and PAS, drugs to which the infection had not been previously exposed. The streptomycin-PAS regimen therefore constituted a more reliable combination for pre- and postoperative treatment than would have been the case had either of these drugs been used earlier in the course. It is also pertinent that, despite the higher incidence of reversal of infectiousness that may be expected from a two-drug regimen in the initial few months, the indications for excisional surgery are not thereby much reduced because the proportion of residual cavities is not greatly diminished. If present concepts change, however, and the "open-negative" case is no longer regarded as one requiring resectional surgery, the higher rate of reversal of infectiousness attainable by initial multiple-drug therapy may become a more important consideration than it appears to be at present. Nevertheless, in present circumstances, with no more satisfactory companion drug now established for general use than either streptomycin or PAS, it is doubtful that one of these should always be used initially with isoniazid. The addition of both together later in the course may be more effective in the end result. Frequently, however, this will be unnecessary, as in the majority of previously untreated cases the attainment of all therapeutic objectives may be expected from the administration of isoniazid alone.

In summary: Thirty-two unselected patients with moderately advanced and far advanced chronic pulmonary tuberculosis were treated with isoniazid alone in daily doses of 10 mg. per kg. of body weight for the initial three weeks of treatment and with 5 mg. per kg. thereafter. In two-thirds of this group the therapeutic objectives were attained without the need of additional medical or surgical therapy. Reversal of infectiousness occurred in 23 of the 29 patients who had tubercle bacilli in the sputum when treatment was started. Cavity closure occurred in 19 of the 30 patients with cavitation at the start of therapy. These results are similar, except for an appreciably lower incidence of reversal of infectiousness, to those observed in a comparable group of patients treated with pyrazinamide-isoniazid.

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HEALTH AND PREVENTIVE MEDICINE,
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CENTER, NEW YORK CITY

August 31, 1957

[7] TUCKER, W. B.: Tr. Fourteenth Conference on Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 41.

[8] JIMENEZ, F. V.: Am. Rev. Tuberc., 1956, 74, 903.

THE INCIDENCE OF CANDIDA ALBICANS IN THE SPUTUM OF TUBERCULOUS PATIENTS

The mechanism by which *Candida albicans*, usually a saprophytic organism, can occasionally become pathogenic, is a problem still unsolved. The apparently increasing number of cases of "moniliasis" described in the recent literature has been explained by some as secondary to the prolonged administration of the so-called "wide spectrum" antimicrobial drugs.[1-3] It is believed that such drugs are capable of changing the normal human flora, thus favoring the emergence of the yeast. This is based on the fact that *Candida albicans* is naturally resistant to almost all antimicrobial drugs now in use.

In this report, results are presented on the incidence of *Candida albicans* in the sputum of tuberculous patients. These results are compared with those reported by other writers in similar studies, carried out before and after the era of antimicrobial therapy.

Two hundred sputum specimens were collected from tuberculous patients of the Chest Department at the General Hospital in Mexico City. The presence of pseudomycelium or yeastlike cells was searched for in each specimen by means of direct examination in Lugol solution, cultures were made streaking a loopful of the material in Sabouraud's glucose agar containing 30 units of penicillin and 50 γ of streptomycin per ml. The cultures were incubated during ten days at 28°C. and were observed daily until the characteristic white creamy colonies of Candida were noticed; strains resembling Candida were transferred to Sabouraud's glucose agar tubes without antimicrobial drugs; these cultures were used for species determination according to the following procedures: Microculture in corn meal agar (Difco) for production of pseudomycelium and chlamydospores;[4] absence of ascospores in carrot plug cultures, and fermentation of several carbohydrates.[5]

Strains showing all of the following features were considered as *Candida albicans*: (*1*) absence of ascospore formation; (*2*) pseudomycelium formation with blastospore clusters and terminal chlamydospores, (*3*) acid and gas production from glucose and maltose, and acid formation or none from sucrose.

The results obtained are shown in table 1. In 9 (4.5 per cent) of 200 sputum specimens examined, yeastlike cells were observed by direct examination; in 46 (23 per cent) the presence of yeasts was demonstrated by culture; 41 (20.5 per cent) of these strains were *Candida albicans*, 1 (0.5 per cent) was identified as *Candida guillermondi*, while the last 4 (2.0 per cent) strains were not Candida.

A comparison of these results with those obtained by some other workers is shown in table 2. Streptomycin began to be used generally in the treatment of clinical tuberculosis in 1948; it can be observed that the frequency of *Candida albicans* in patients with pulmonary tuberculosis before and after the period does not differ considerably.

Recently, Helms[6] reported a very high percentage of sputum specimens positive for *Candida albicans* in 205 unselected patients. In his series, however, there were only 8 tuberculous patients, 6 of whom had *Candida albicans* in the sputum. Although this is a very small number, Helms found the yeast in the sputum of many patients with other diseases (70 to 80 per cent). This is the highest percentage reported so far and it is difficult

[1] Wolff, F. W.: Lancet, 1952, *262*, 1236.
[2] Torack, R. M.: Am. J. Med., 1957, *22*, 872.
[3] Young, G., *et al.*: J. Bact., 1956, *72*, 525.
[4] Riddell, R. W.: Mycologia, 1950, *42*, 265.
[5] Martin, D. S., *et al.*: J. Bact., 1937, *34*, 99.
[6] Helms, P.: J. Clin. Path., 1956, *9*, 372.

TABLE 1

INCIDENCE OF CANDIDA ALBICANS IN THE SPUTUM OF TUBERCULOUS PATIENTS*

Samples Examined		Samples in Which Yeast-like Cells Were Observed by Direct Examination		Samples in Which Yeasts Were Isolated by Culture		Samples in Which the Isolated Yeast Was *Candida albicans*	
Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Per Cent
200	100	9	4.5	46	23	41	20.5

*Each sample obtained from a different patient.

TABLE 2

OCCURRENCE OF CANDIDA ALBICANS IN TUBERCULOUS SPUTUM ACCORDING TO SEVERAL AUTHORS

Author and Year	Number of Sputa Examined	Per cent of Sputum with *Candida albicans*	Reference Footnote
Fisher and Arnold, 1936	160	13.0	8
Weedon *et al.*, 1937	55	9.0	9
Burt and Ketchum, 1941	693	36.0	10
Schwarting and Skinner, 1948	500	17.0	7
Brygoo, 1951	102	20.0	11
Rioux *et al.*, 1954	266	24.0	12
This paper	200	20.5	

to accept, not only in view of all other reports, but also because of the result of unpublished observations, in which *Candida albicans* was carefully searched for in the sputum of 40 healthy adults without success.

However, Schwarting and Skinner[7] have explained the different results obtained by several writers suggesting that an unequal incidence of the yeast could be found in different population groups and also by the dissimilar methods employed in the species determination.

It was not possible to establish the diagnosis of bronchopulmonary moniliasis in any of the present cases despite the presence of yeastlike cells and the isolation of *Candida albicans* by culture from the sputum of some patients. This suggests that in such patients the yeasts were present as saprophytes either in the mouth or in the lower respiratory tract without causing clinical symptoms.

It may be concluded that streptomycin, the antimicrobial drug most frequently used in Mexico in the therapy of tuberculosis, apparently does not influence the incidence of *Candida albicans* in the sputum of these patients.

In summary: Two hundred sputum samples from tuberculous patients were examined for *Candida albicans*. Direct examinations, cultures, and fermentation tests of several carbohydrates revealed an incidence of 20.5 per cent of *Candida albicans*. These results do

[7] SCHWARTING, V. M., AND SKINNER, C. E.: Mycopathol., 1949, *4*, 349.
[8] FISHER, C. V., AND ARNOLD, L.: Univ. Illinois Bull., 1936, *33*, 1.
[9] WEEDON, F. R., *et al.*: J. Bact., 1937, *34*, 657.
[10] BURT, K. L., AND KETCHUM, H. M.: Am. J. Trop. Med., 1941, *21*, 427.
[11] BRYGOO, E. R.: Ann. Inst. Pasteur, 1951, *81*, 676.
[12] RIOUX, J. A., *et al.*: Poumon, 1954, *10*, 101.

not differ considerably from those obtained by other workers before the era of antimicrobial therapy. It is concluded that streptomycin, an antimicrobial frequently used in the treatment of tuberculosis, does not affect the incidence of *Candida albicans* in the sputum of treated patients.

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October 23, 1957

THE DOSE OF HISTOPLASMIN H-42 FOR SKIN TESTING

It became apparent a little more than ten years ago that a benign form of histoplasmosis was widely prevalent in some parts of the United States. As a consequence, the Public Health Service anticipated that extensive skin testing with histoplasmin would be required for studying the epidemiology of histoplasmosis as well as for the differential diagnosis of tuberculosis, histoplasmosis, and other fungal infections. As a considerable amount of histoplasmin would be needed for those purposes it was believed that there would be advantages if a single large lot of the antigen could be prepared, standardized, and made available to responsible investigators. Thus, in 1947, Dr. Arden Howell, Jr. of the Tuberculosis Division of the Public Health Service prepared several batches of histoplasmin, using a method similar to that described earlier by Emmons and associates,[1] and three of those batches were pooled to form one large lot identified as H-42. This product was "standardized," as reported in a previous paper,[2] by relating its potency to other histoplasmin products for which an appropriate dose had been determined empirically. The standardization, based on comparative intracutaneous tests in more than 12,000 persons, led to the acceptance of a dose of 0.1 ml. of a 1:100 dilution of the preparation designated as "stock" H-42 as satisfactory for skin-testing studies.

The Tuberculosis Division began using lot H-42 for some of its field studies in 1949 and since 1952 lot H-42 has been used extensively by the Division and its collaborators both in the United States and abroad.[3] It was also in 1952 that the Biologics Control Laboratory of the National Institutes of Health requested and was given approximately 500 ml. of stock H-42 to use as a reference product for the standardization of histoplasmin from other sources. Several hundred thousand tests have already been given with lot H-42, and the amount still on hand is sufficient, if carefully preserved and not wasted, for several million more tests. As far as can be judged, the stock antigen has lost none of its potency since it was prepared ten years ago.

Recently, in reviewing material from histoplasmin skin-testing surveys carried out during the last decade, it appeared that a slightly stronger concentration than the 1:100 dilution in stock H-42 (or its equivalent) might have given more precise results. It therefore seemed advisable to make a field comparison of the 1:100 dilution with a more concentrated dilution, and advantage was taken of an opportunity to do so in the spring of 1957 in a skin-testing survey of high school students in Whitfield and Murray Counties, Georgia.[4]

Nearly 90 per cent of the enrollment of five high schools in the two counties, totaling more than 2,000 students, were given intracutaneous tests with either a 1:100 dilution of lot H-42 or double that concentration, a 1:50 dilution. The two dilutions were alternated as the students came to be tested; and, to prevent observer bias, the person who measured the sizes of the reactions two days later was not allowed to know what dilution had been used for a particular test. As the groups of students tested with the two dilutions should be entirely comparable, differences in results can be ascribed to differences between the two dilutions.

Results are shown in figure 1 for the 1,018 white students tested with the 1:100 dilution and the 995 tested with the 1:50 dilution. For both doses, the distributions of the sizes of

[1] Emmons, C. W., *et al.*: Pub. Health Rep., 1945, *60*, 1383.
[2] Shaw, L. W., *et al.*: Pub. Health Rep., 1950, *65*, 583.
[3] Edwards, P. Q., and Kiaer, J. H.: Am. J. Trop. Med., 1956, *5*, 235.
[4] Edwards, P. Q., *et al.*: Dis. of Chest, 1957 (in press).

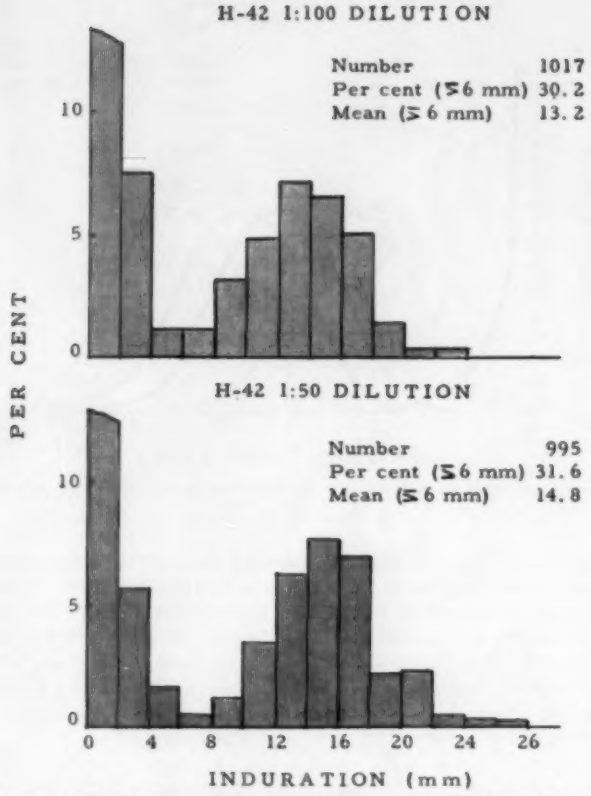

FIG. 1. Frequency distributions of sizes of reactions to two dilutions of histoplasmin lot H-42 among white high school students, Whitfield and Murray Counties, Georgia, March, 1957.

the reactions are bimodal: each shows a separation into two groups, one composed of reactions measuring approximately 6 mm. or more in diameter; the other, of reactions measuring less than 6 mm. The group of large reactions is distributed in a form resembling the normal probability frequency curve. As some persons in the population are assumed to have been infected with Histoplasma, and as those persons can be expected not only to react to histoplasmin but to have reactions distributed in the form of a normal curve, it may be inferred that the group of large reactions represents the infected (positive) segment of the population. Conversely, those who have not been infected would be expected to have no reaction to histoplasmin or, because of observational error, only a small reaction.

The difference between the results for the 1:100 and 1:50 dilutions corresponds surprisingly well with that to be expected from two slightly different doses of the same product. First, the sizes of the negative reactions are not affected to any appreciable degree by increasing the dose of the antigen. Second, the positive reactions are approxi-

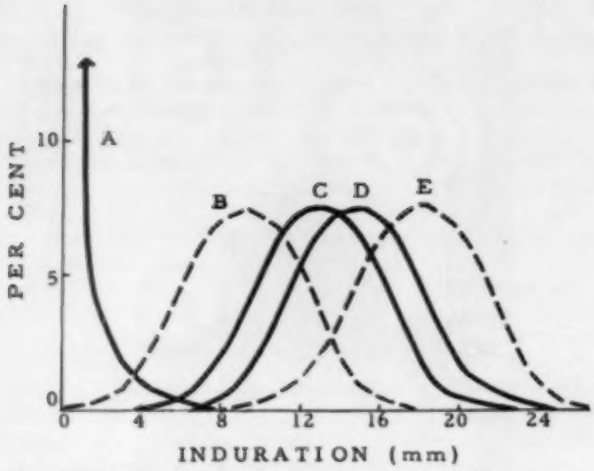

FIG. 2. Schematic distributions of sizes of reactions to four different concentrations of the same antigen.

mately 1.5 mm. larger in average size with the larger dose, and the entire positive group is thereby moved farther away from the negative group. In other words, by doubling the dose of histoplasmin, the positive reactions are separated more clearly from the negative reactions by reducing the amount to which the positive group overlaps the negative group. This has distinct advantages. The fewer the reactions that overlap, the greater the accuracy of classifying reactions as positive or negative because the two groups are better separated and the effect of observational error is reduced. Previous reports have dealt with this same problem in connection with tuberculin testing.[5, 6]

A schematic interpretation of the results is given in figure 2, in which curve *A*, at the left, represents the distribution of negative reactions and curves *C* and *D* have been drawn to correspond roughly to the distributions of positive reactions to the 1:100 and 1:50 dilutions. Curves *B* and *E* represent theoretical results with a weaker and stronger dose of the same product. The weakest dose, *B*, gives a positive group that centers at 10 mm. and overlaps with the negative group from zero to almost 8 mm. The next stronger dose, *C*, moves the positive group toward the right and thereby appreciably reduces the amount of overlapping with the negative group. *D*, in turn, further reduces the amount of overlap and with *E*, the strongest dose, it is negligible.

With each successive increase in the dose of histoplasmin, however, the frequency of very large reactions increases. For purely practical reasons, the frequency of large reactions must be kept within tolerable limits in human skin-testing work. In the present study, the proportion of positive reactions measuring 20 or more mm., for example, increased from 0.8 per cent with the 1:100 dilution to 3.4 per cent with the 1:50. Reactions with vesicles were rare with both doses, there being only 3 in each group, and none of the reactions showed necrosis. While a few of the students with very large reactions inquired about their significance, there were no complaints.

[5] PALMER, C. E., *et al.*: Lancet, 1954, *2*, 1274.

[6] WHO TUBERCULOSIS RESEARCH OFFICE: Bull. World Health Org., 1955, *12*, 63 and 169.

TABLE 1

PULMONARY CALCIFICATION/INFILTRATION ACCORDING TO SIZE OF REACTION TO HISTOPLASMIN H-42 AMONG WHITE HIGH SCHOOL STUDENTS, WHITFIELD-MURRAY COUNTIES, GEORGIA

Size of Reaction to Histoplasmin (In millimeters)	Dilution of Histoplasmin				Total	
	1:100		1:50		Both Dilutions	
	Number Tested	Number with Calcification/ Infiltration	Number Tested	Number with Calcification/ Infiltration	Number Tested	Number with Calcification/ Infiltration
0–1	623	2	605	4*	1228	6*
2–3	76	—	57	—	133	—
4–5	12	—	17	—	29	—
6–7	13	2	5	1	18	3
8–9	33	3	12	—	45	3
10–11	50	3	35	5	85	8
12–13	72	15	63	11	135	26
14–15	66	10	76	9	142	19
16–17	51	8†	69	4	120	12†
18–19	14	2	22	2	36	4
20–21	4	1	24	—	28	1
22–23	4	—	5	—	9	—
24–25	—	—	3	—	3	—
26–27	—	—	2	—	2	—
Total.....	1018	46	905	36	2013	82

* Includes two persons with reactions of 20 and 21 mm. to tuberculin, 5 TU

† Includes one person with a reaction of 18 mm. to tuberculin, 5 TU

The efficiency of a test in separating a population into groups, regardless of the precision of the separation, has little meaning unless the test is also specific. In this instance, one group should contain all or nearly all persons infected with Histoplasma; and the other group, all or nearly all who have not been infected. The specificity of a test must be evaluated of course by criteria of infection which are independent of the response to the test itself.

Roentgenographic evidence of pulmonary infiltrations and/or calcifications, while admittedly crude, is one of the few criteria of infection with Histoplasma that can be used to evaluate the specificity of the histoplasmin test in human populations.[7-9] If other causes of pulmonary lesions can be identified in the population, then the frequency of lesions in relation to the sizes of the skin reactions may be analyzed to test the assumption that the two groups distinguished by the skin test correspond to the infected and uninfected members of the population.

In the present study, 70 mm. photofluorograms of the chest were taken immediately before the skin tests were given and were read independently by two physicians without knowledge of the skin-test reactions. The results given in table 1 show that pulmonary findings interpreted as either calcification or infiltration by both readers were highly

[7] PALMER, C. E.: Pub. Health Rep., 1945, *60*, 513.

[8] CHRISTIE, A., AND PETERSON, J. C.: Am. J. Pub. Health, 1945, *35*, 1131.

[9] GODDARD, J. C., *et al.*: Pub. Hea'th Rep., 1949, *64*, 820.

concentrated in the group identified by the test as positive reactors. Thus, only 6 (0.4 per cent) of 1,390 students in the negative group had pulmonary findings; and 2 of those 6 had strong tuberculin reactions. Among the 623 with positive reactions, 76, or more than 12 per cent, had pulmonary findings and only one of those 76 also reacted to tuberculin.

The roentgenographic findings thus support the interpretation of the two groups distinguished by the histoplasmin test as representing the infected and uninfected persons in the population. And, as the 1:50 dilution appears to separate the two groups more efficiently than the 1:100 dilution, without surpassing the tolerable limit of large or severe reactions, it would appear to be a more useful dose for both epidemiologic and clinical purposes.

The material presented here is intended to illustrate only one admittedly limited way of evaluating the efficiency of a skin test. Moreover, as the results refer to the product H-42, they should not be construed as applicable to other histoplasmin products. It should be emphasized also that the present study was done in an area where sensitivity to histoplasmin does not appear to be affected by cross-sensitization resulting from infection with other organisms, as is the case in some parts of the country where many histoplasmin reactions can be ascribed to infection with Coccidioides or perhaps other fungi.[10] Until further studies have been done in other areas, therefore, the 1:50 dilution of lot H-42 cannot be judged as generally more satisfactory than the 1:100 dilution.

TUBERCULOSIS PROGRAM
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November 4, 1957

[10] PALMER, C. E., *et al.*: Am. J. Hyg., 1957, *66*, 169.

Books

CLINICAL CARDIOPULMONARY PHYSIOLOGY. Edited by Burgess L. Gordon. Pp. 759, Grune and Stratton, Inc., New York, 1957. Price, $15.75.

As indicated by the editor in the foreword of this book, the preparation of a monograph on clinical cardiopulmonary physiology " . . . is a big order . . . " It is so large as to be an almost prohibitive task for a single author. Hence, the present attempt to fill the order is under the direction of a nine-man editorial board aided by 43 additional authors.

It is unfortunate that the central purpose of the book—the description and interpretation of physiologic phenomena encountered in patients with disease of the heart or lungs—is obscured periodically as the reader progresses from cover to cover. In some chapters, purely clinical, etiologic, or detailed therapeutic considerations are permitted to dominate with minimal reference to disturbances in function. In the case of a disease (or group of related diseases) on which physiologic data are meager, this may be justified, although the wisdom of devoting to such a condition an entire chapter of a book on *function* is questionable. However, in the case of a condition about which a large body of sound physiologic information is available in the literature, the failure to present the available facts appears inexplicable. This deficiency is most notable in the sections on disturbances in cardiovascular function, which have been covered desultorily and inadequately. (Example: The newer information on the genesis and clinical and physiologic significance of cardiovascular sounds is not presented.) Some of the repetitious chapters on pulmonary disease could have been combined or even jettisoned to provide room for a more adequate cardiovascular section.

Multiple authorship is responsible for a number of additional defects. These include an uneveness in style and clarity and failure in occasional chapters to make effective use of illustrations or to convey to the reader in tabular or other form a clear notion of the magnitude of alterations in function which may be encountered in the diseases under consideration. In view of the quantitative nature of physiology, the importance of the latter is evident.

The reviewer hastens to indicate that a number of the chapters (including some which contain little or no phyiologic information) have been written with obvious care and organization. The discerning and critical student of this field will recognize these chapters readily and profit from reading them.

As the American College of Chest Physicians and its Editorial Board recognize, there is a need for assembling and organizing the extensive observations on the function of the human heart and lungs to facilitate their application in clinical practice. The volume they offer only partially accomplishes this objective. It is difficult to avoid contrasting the pulmonary section of this 758-page tome with the lucid, terse, beautifully illustrated 219-page volume, *The Lung. Clinical Physiology and Pulmonary Function Tests* by Comroe and his associates.

DANIEL S. LUKAS
New York, New York

HALSTED OF JOHNS HOPKINS. By Samuel James Crowe. Pp. 247. Charles C Thomas, Springfield, Illinois, 1957. Price, $5.00.

The impact of The Johns Hopkins Medical School on medical education has often been related, but it is doubtful if one can find a more effective account than the late Dr. Crowe

has provided in this small volume. Dr. Crowe, son of a physician, entered medical school at Johns Hopkins with reluctance. However, a chance encounter with Dr. Halsted changed his outlook from one of distaste for medicine to one of enthusiasm. After graduation, he became a member of the surgical resident staff which led to an opportunity to go to Harvard with Cushing. Halsted persuaded young Crowe to remain at Hopkins to form the Department of Otolaryngology, a specialty with which Crowe was totally unfamiliar and in which he had a complete lack of experience. Dr. Crowe accepted the challenge and became one of the great names in this division of surgery.

Dr. Crowe is eminently qualified to record the signal accomplishments of the group presented in this book. He has chosen a remarkably effective method for the description of Halsted and his manners and methods. Rather than a long chronological account of the life and accomplishments of the great surgeon, he has provided a brief but lucid story of the founding of the medical school. This is followed by an account of the early days of Halsted at the new institution. Dr. Crowe then turns to the men whom Halsted chose to aid him in the development of various phases of surgery at Hopkins. For this purpose he selected the following: Cushing, Dandy, Mitchell, Young, Baer, Baetjer, and Crowe. Through the chapters devoted to each of them an effective picture is gained of the system of training that Halsted developed and his selection of some of these men to organize new divisions of surgical specialities and radiology. Halsted's application of laboratory investigation of new methods prior to clinical trial is forcefully indicated.

The reader's interest is stimulated by the intimate relationship that existed between the author and the subjects of the volume. There are a number of appropriate illustrations; and the book has a most felicitous foreword by Dr. Blalock.

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New York, New York

BOOKS RECEIVED

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American Trudeau Society

Medical Section of the National Tuberculosis Association

PRELIMINARY PROGRAM, MEDICAL SESSIONS, AMERICAN TRUDEAU SOCIETY ANNUAL MEETING

MONDAY, MAY 19

10:00 a.m.–12:15 p.m.

1A Tuberculosis Including Chemotherapy

Follow-up of Patients Observed in Previous USPHS Tuberculosis Therapy Trials—CARROLL E. PALMER, M.D., Washington, D. C.

Kanamycin in Experimental Guinea Pig Tuberculosis—KEN YANAGISAWA, M.D., and KOOMI KANAI, M.D., Tokyo, Japan

The Antitubecurlous Activity of Kanamycin in Vitro and in the Experimental Animal (Guinea Pig)—WILLIAM STEENKEN, JR., D.Sc., VINCENT MONTALBINE, and J. R. THURSTON, Saranac Lake, N. Y.

Kanamycin: A New Tuberculostatic Agent—KENNETH WRIGHT, M.D., ATTILIO RENZETTI, M.D., JOSEPH LUNN, B.S., PAUL BUNN, M.D., DONALD JOHNSON, and OLGA KRAJNJAK, Syracuse, N. Y.

Corticosteroids in the Treatment of Tuberculosis—HASKELL J. WEINSTEIN, M.D., and JOHN J. KOLER, M.D., Seattle, Wash.

The Effect of Increasing Oral Isoniazid Dosage on Biologically Active and Total Isoniazid Serum Levels—CAPT. ROBERT L. TAYLOR (MSC), O. L. WEISER, M. A., COL. RICHARD S. FRASER (MC), COL. JAMES A. WIER (MC), and LT. COL. RICHARD R. TAYLOR (MC) Denver, Colo.

Cycloserine and Viomycin in the Treatment of Pulmonary Tuberculosis—WILLIAM S. SCHWARTZ, M.D., and R. E. MOYER, M.D., Oteen, N. C.

Pregnancy Following Major Thoracic Surgery for Tuberculosis—GEORGE SCHAEFER, M.D., R. GORDON DOUGLAS, M.D., and STANLEY J. BIRNBAUM, M.D., New York, N. Y.

Tuberculous Polyserositis—CAPT. C. G. SPIVEY, MC, USAF, and COL. J. A. WIER, MC, USA, Denver, Colo.

Pelvic Tuberculosis in the Female—ROBERT R. HENDERSON, M.D., JULIA M. JONES, M.D., and GORDON W. DOUGLAS, M.D., New York, N. Y.

10:00 a.m.–12:15 p.m.

1B Physiology

Respiratory Function in Pulmonary Tuberculosis—DAVID G. SIMPSON, M.D., JOHN H. MCCLEMENT, M.D., and ANDRÉ COURNAND, M.D., New York, N. Y.

The Prevalence of Diffuse Airway Obstruction in Men Between 40 and 65 Years of Age—GEOFFREY L. BRINKMAN, M.D., and E. OSBORNE COATES, JR., M.D., Detroit, Mich.

Observations on the Circulatory Dynamics in Patients with Chronic Lung Diseases—K. H. KILBURN, M.D., and H. O. SIEKER, M.D., Durham, N. C.

Total Body Potassium and Sodium and Extracellular Fluid in Chronic Pulmonary Insufficiency—GEORGE L. BAUM, M.D., ARTHUR KAUPE, M.D., JOSE D. CARBALLO, M.D., and MORRIS M. DICK, M.D., Coral Gables, Fla.

Cystic Disease of the Lungs—GUSTAVE A. LAURENZI, M.D., GERARD M. TURINO, M.D., and ALFRED P. FISHMAN, M.D., New York, N. Y.

Relationship Between Airway Resistance and the State of Inflation of the Lung in Normal Subjects and in Patients with Obstructive Disease—T. O. CHENG, M.D., M. P. GODFREY, M.B., and R. H. SHEPARD, M.D., Baltimore, Md.

The Work of Breathing with Increased Airway Resistance and Intermittent Positive Pressure Assistance—R. H. JONES, M.D., J. MACNAMARA, M.D., M. F. ELLICOTT, M.D., and E. A. GAENSLER, M.D., Boston, Mass.

Some Aspects of the Pulmonary Diffusion Capacity for Carbon Monoxide—M. MOSTAFA ABDEL-FATTAH, M.D., MARGARET E. GANTT, M.D., and DAVID W. CUGELL, M.D., Chicago, Ill.

Relationship Between Pulmonary Capillary Blood Flow, Pulmonary Diffusing Capacity, and Pulmonary Capillary Blood Volume at Rest and Exercise—W. S. SPICER, JR., M.D., R. L. JOHNSON, JR., J. M. BISHOP, and R. E. FORSTER, M.D., Philadelphia, Pa.

12:30 p.m. –1:45 p.m.

Sandwich Seminar I*

Drugs in the Management of Psychiatric Problems Encountered in Tuberculous Patients—EMIL ROTHSTEIN, M.D., Brockton, Mass., *Moderator*

Participants to be announced.

Sandwich Seminar II*

Failure of Therapy in Pulmonary Tuberculosis—NICHOLAS D. D'ESOPO, M.D., West Haven, Conn., *Moderator*

Participants to be announced.

Sandwich Seminar III*

Problems in the Pathogenesis of Pulmonary Emphysema—ROGER S. MITCHELL, M. D., Denver, Colo. *Moderator*. Participants to be announced.

2:00 p.m.–3:30 p.m.

The Changing Status of the Tuberculin Test (Joint Medical, Public Health, and Nursing Session)

KARL PFUETZE, M.D., Chicago, Ill., *Moderator*

Panelists: GEORGE COMSTOCK, M.D., Washington, D. C.; KATHARINE H. K. HSU, M.D., Houston, Texas; JULIA M. JONES, M.D., New York, N. Y.; ARTHUR B. ROBINS, M.D., New York, N. Y.

3:45 p.m.–5:30 p.m.

2A Surgery

Pleural Tuberculosis: A Clinicopathological Study—BELLA S. STRAUSS, M.D., WILLIAM E. LORING, M.D., and ROBERT HENDERSON, M.D., New York, N. Y.

An Analysis of Preoperative Bronchial Biopsy in 100 Patients with Pulmonary Tuberculosis—NEIL C. ANDREWS, M.D., CLARENCE I. BRITT, M.D., and PHILIP C. PRATT, M.D., Columbus, Ohio

*Each Seminar will be limited to 30 physicians in order to allow for informal discussion with the experts. Tickets must be purchased in advance from the ATS. Locations for all Seminars to be announced.

Pneumonectomy in the Treatment of Pulmonary Tuberculosis—T. W. SHIELDS, M.D., W. M. LEES, M.D., and R. T. FOX, M.D., Chicago, Ill.

The Collapse Therapy of Pulmonary Tuberculosis by Means of Extraperiosteal Plombage—THOMAS F. BOYD, M.D., EUGENE G. LAFORET, M.D., and JOHN W. STRIEDER, M.D., Boston, Mass.

Resectional Therapy for Open-Negative Tuberculous Lesions—WATTS R. WEBB, M.D., and JESSE L. WOFFORD, M.D., Jackson, Miss.

Tracheal Fenestration as a New Method of Treatment for Advanced Emphysema—EDWARD E. ROCKEY, M.D., SAMUEL A. THOMPSON, M.D., CHARLES F. BLAZSIK, M.D., EDGAR MAYER, M.D., and ISRAEL RAPPAPORT, M.D., New York, N. Y.

Reconstructive Surgery of the Trachea and Bronchi—OSLER A. ABBOTT, M.D., and EVERTON SANTOS, M.D., Emory University, Ga.

The Value and Risks of Modified Needle Biopsy for Undiagnosed Lung Lesions—VINCENT W. LAUBY, M.D., W. EMORY BURNETT, M.D., GEORGE P. ROSEMOND, M.D., H. TAYLOR CASWELL, M.D., and R. ROBERT TYSON, M.D., Philadelphia, Pa.

3:45 p.m.–5:30 p.m.

2B Immunity in Tuberculosis

Cellular Immunity in Tuberculosis: A Critical Evaluation of Some Observations—EMANUEL SUTER, Ph.D., Gainesville, Fla.

Passive Transfer of Protective Humoral Antibodies in Tuberculosis—CHARLOTTE MARKER ZITRIN, M.D., New York, N. Y.

The Interplay of an Immune Substance (Possibly Lysozyme) with Tuberculopolysaccharide and Its Antibody in Tuberculosis—FLORENCE B. SEIBERT, Ph.D., Philadelphia, Pa.

The Relationship of Certain Factors to Necrosis in Guinea Pig Tuberculin Reactions—CUTTING B. FAVOUR, M.D., Palo Alto, Calif.

Globulin Fractions as Related to Immunity—IRENE MELVIN, M.A., and H. M. VANDIVIERE, M.S., III, Chapel Hill, N. C.

Immunogenic Considerations of Several Antituberculosis Vaccines—H. STUART WILLIS, M.D., H. M. VANDIVIERE, M.S. III, and MARGARET R. VANDIVIERE, M.A., Chapel Hill, N. C.

Discussion to be opened by ESMOND R. LONG, M.D., Pedlar Mills, Va.

4:15 p.m.–5:15 p.m.

Demonstration I

Cardiac Research Unit, Philadelphia General Hospital, Blockley Division—SAMUEL BELLETT, M.D., and STAFF.

The following areas will be covered: vector cardiography, electrocardiography, hemodynamics, electrolytes, angiocardiography, and congenital heart disease.

This demonstration is limited to 50 physicians. Reservations must be secured in advance from the ATS.

Demonstration II

Heart Lung Machine, Jefferson Medical College—JOHN H. GIBBON, JR., M.D., and STAFF

Limited to 50 Physicians. Reservations must be secured in advance from the ATS.

TUESDAY, MAY 20

9:00 a.m.–10:30 a.m.

3A Symposium on Sarcoidosis

Epidemiology—JOHN S. CHAPMAN, M.D., Dallas, Texas

Relationship Between Tuberculosis and Sarcoidosis—MAX MICHAEL, JR., M.D., Brooklyn, N. Y.

Sarcoid Combinations: Coexisting Sarcoidosis and Chronic Infections—J. RICHARD JOHNSON, M.D., Madison, Wis.

Newer Diagnostic Methods—HAROLD L. ISRAEL, M.D., Philadelphia, Pa.

Prednisone and Prednisolone Therapy—LOUIS E. SILTZBACH, M.D., MARK M. IMBERMAN, M.D., and HOWARD GROSSMAN, M.D., New York, N. Y.

9:00 a.m.–10:45 a.m.

3B Laboratory Research Including Tuberculosis

On the Role of the Thyroid in Native Resistance to Tuberculosis—MAX B. LURIE, M.D., PETER ZAPPASODI, B.S., RICHARD S. LEVY, M.S., and ROBERT G. BLAKER, M.A., Philadelphia, Pa.

Sonic Fragility of Leukocytes from Guinea Pigs Vaccinated with BCG—ROBERT A. PATNODE, Ph.D., and PAUL C. HUDGINS, Washington, D. C.

Serum Concentrations and Urinary Excretion of INH, Streptomycin and PAS and Their Metabolites, as They Affect Their Antituberculous Efficacy—ROGER S. MITCHELL, M.D., DICK K. RIEMENSNIDER, and J. CARROLL BELL, M.D., Denver, Colo.

Pathogenicity and Cord Formation Titration—H. M. VANDIVIERE, M.S. III, IRENE MELVIN, M.A., and H. E. KENNEDY, Ph.D., Chapel Hill, N. C.

The Pathogenicity of Isoniazid-Resistant, Catalase-Negative Tubercle Bacilli for the Silicotic Host (Guinea Pig)—WILLIAM STEENKEN, JR., D.Sc., Saranac Lake N. Y.; JAMES W. RALEIGH, M.D., Sunmount, N. Y.; D. H. CHAPMAN, Sunmount, N. Y.; and M. M. SMITH, Saranac Lake, N. Y.

Temporary Lung Density Increases in Immunized Mice Resembling Delayed Hypersensitivity Reactions—ALFRED J. CROWLE, Ph.D., Denver Colo.

Biochemistry of the Lung: Some Aspects of Glucose Metabolism in Normal Lung—VERNE D. HOSPELHORN, Ph.D., and MARTIN J. FITZPATRICK, M.D., Kansas City, Kans.

Pleural Fluid Amylase in Pancreatitis and Other Diseases—JAMES F. HAMMARSTEN, M.C., BARNEY LIMES, M.D., and WALTER HONSKA, JR., M.D., Oklahoma City, Okla.

11:00 a.m.–12:00 p.m.

The Amberson Lecture—ESMOND R. LONG, M.D., Pedlar Mills, Va.

12:30 p.m.–2:00 p.m.

American Trudeau Society Luncheon and Business Meeting

2:45 p.m.–4:00 p.m.

Radiation Safety (Joint Medical, Public Health, and Nursing Session)

RICHARD CHAMBERLAIN, M.D., Philadelphia, Pa., *Moderator*

Panelists: Internist—THEODORE L. BADGER, M.D., Boston, Mass.; *Tuberculosis Control Officer*—PAUL T. CHAPMAN, M.D., Detroit, Mich.; *Physicist*—ROBERT O. GORSON, Philadelphia, Pa.

4:15 p.m.–5:45 p.m.

4A Symposium on Fungus Diseases

Histoplasmosis

Pathogenesis—John J. Procknow, M.D., Malcolm I. Page, M.D., and Clayton G. Loosli, M.D., Chicago, Ill.

Course and Treatment—Harry Rubin, M.D., Kansas City, Kan.; J. Lewis Yates, M.D., Charles A. Brasher, M.D., Mt. Vernon, Mo., Howard W. Larsh, Ph.D., and Michael L. Furcolow, M.D., Kansas City, Kan.

Blastomycosis Therapy—E. Richard Herrell, M.D., Ann Arbor, Mich.

Coccidioidomycosis: Advances in Treatment—William Winn, M.D., Springville, Calif.

Crytococcosis, an Increasing Problem—John S. Chapman, M.D., Dallas, Texas.

Pathology of Fungus Disease—Henry C. Sweany, M.D., Mount Vernon, Mo.

Cultural Characteristics of Systemic Fungi and Methods for Evaluating Therapeutic Agents—Howard W. Larsh, Ph.D., Norman, Okla.

4:15 p.m.–5:45 p.m.

4B Atypigal Mycobacteria

Purified Protein Derivatives (PPD) and Other Antigens Prepared from Acid-fast Bacilli and Nocardia asteroides—Lewis F. Affronti, M.A., Philadelphia, Pa.

Results of Testing with Tuberculins Prepared from Atypical Acid-Fast Organisms—Carroll E. Palmer, M.D., Washington, D. C.

The Correlation of the Reaction to Protein from Chromogenic Mycobacteria to the Reaction to Tuberculin (OT)—Joseph D. Aronson, M.D., Philadelphia, Pa.; William Kraus, M.D., Harrisburg, Pa.

Clinical Evaluation of 100 Patients with Chronic Pulmonary Disease Due to, or Associated with, Atypical Mycobacteria—Albert G. Lewis, Jr., M.D., Tampa, Fla.; Roberts Davies, M.D., Tallahassee, Fla.; Eunice M. Lasche, M.D., Tampa, Fla.; Ernest N. Lerner, M.D., Tampa, Fla.; Albert V. Hardy, M.D., James O. Bond, M.D., Jacksonville, Fla.; Frank P. Dunbar, Tampa, Fla.

The Production of Bacteriocin-like Substances by Strains of Atypical Mycobacteria—Emilo C. Mora, M.S., A. Eisenstark, Ph.D., Manhattan, Kan.

Quantitative Aspects of Neutral Red Reaction of Typical and "Atypical" Mycobacteria—Lawrence G. Wayne, Ph.D., San Fernando, Calif.

4:15 p.m.–5:15 p.m.

4C Joint Medical and Nursing Session

Out-Patient Care of Tuberculosis—Frances S. Lansdown, M.D., Julia M. Jones, M.D., and Fannie Behlen, R.N., New York, N. Y.

Social Attitudes of the Tuberculous Alcoholic and their Bearing on Hospital Adjustment—Kate L. Kogan, Ph.D., Seattle, Wash.

The Tuberculous Alcoholic Before and During Hospitalization—Emily B. Fergus, M.D., and Joan K. Jackson, Ph.D., Seattle, Wash.

Control of the Recalcitrant Tuberculous Patient—J. P. Myles Black, M.D., Olive View, Calif.

4:15 p.m.–5:15 p.m.

Demonstration III

Demonstration of Cardiac Ventriculography and Suprasternal Transthoracic Aortograph, Department of Radiology, Hahnemann Medical College—J. Stauffer Lehman, M.D., Philadelphia, Pa., *Moderator*

This demonstration is limited to 6 physicians. Reservations must be secured in advance from the ATS.

Demonstration IV

Newer Developments in Radiologic Diagnosis of Chest Diseases, Department of Radiology, Hospital, University of Pennsylvania—EUGENE P. PENDEGRASS, M.D., and STAFF.

WEDNESDAY, MAY 21

9:00 a.m.–10:30 a.m.

5A Pulmonary Diseases in Children

A Report of 105 Patients with Cystic Fibrosis of the Pancreas Studied Over a 5–14 Year Period—HARRY SHWACHMAN, M.D., and LUCAS L. KULCZYCKI, M.D., Boston, Mass.

Panel on Cystic Fibrosis of the Pancreas

JOSEPH ATKINS, M.D., Philadelphia, Pa., *Moderator*

Panelists: JAMES B. AREY, M.D., JOHN HOPE, M.D., and GUILIO BARBERO, M.D., Philadelphia, Pa.; HARRY SHWACHMAN, M.D., Boston, Mass.

Adrenal Corticosteroids in the Treatment of Childhood Tuberculosis—MARGARET H. D. SMITH, M.D., J. C. CARDONA, M.D., N. MATSANIOTIS, M.D., H. LICHTER, M.D., and S. STONE, M.D., New York, N. Y.

Experience with the Heaf Test in Children—MARGARET H. D. SMITH, M.D., and SHIRLEY ANDERSEN, New York, N. Y. To be presented by SHIRLEY ANDERSEN.

The Application of Image Intensification and Cine Fluorographic Technics to the Study of Chest Disease in Infancy—JOHN A. KIRKPATRICK, JR., M.D., and C. ROBERT E. WELLS, M.D., Philadelphia, Pa.

Diagnostic and Therapeutic Considerations Relative to Lobar Emphysema of Infancy—OSLER A. ABBOTT, M.D., and WILLIAM E. VAN FLEIT, M.D., Emory University, Ga.

9:00 a.m.–10:30 a.m.

5B Cardiovascular Disease

The Current Status of Open Heart Surgery—DWIGHT MCGOON, M.D., Rochester, Minn.

A Rational Clinical Approach to Traumatic Thoracic Aortic Aneurysms—BEN EISEMAN, M.D., and WILLIAM G. RAINER, M.D., Denver, Colo.

Coronary Arteriography—J. STAUFFER LEHMAN, M.D., Philadelphia, Pa.

Relationship of Physical Activity to the Development of Coronary Atherosclerosis and Sudden Death from Coronary Occlusion—DAVID M. SPAIN, M.D., Brooklyn, N. Y.

11:00 a.m.–12:30 p.m.

6A Nontuberculous Diseases of the Lungs

Pulmonary Glycoproteinosis: A New Entity—SAMUEL H. ROSEN, AVERILL A. LIEBOW, and BENJAMIN CASTLEMAN, New Haven, Conn.

Intrathoracic Complications of Subdiaphragmatic Infection—DAVID P. BOYD, M.D., Boston, Mass.

Pulmonary Capillary Proliferation Induced by Oxygen Inhalation—PHILLIP C. PRATT, M.D., Columbus, Ohio.

Bronchogenic Carcinoma

Lung Cancer in Women—HELEN PERCY, M.D., and KATHARINE R. BOUCOT, M.D., Philadelphia, Pa.

Experimental Lung Cancer—MARVIN KUSCHNER, M.D., New York, N. Y.

Tumor Cells in Blood in Bronchogenic Carcinoma—C. A. ROSS, M.D., Buffalo, N. Y.

Newer Morphologic Approaches to Prognosis in Lung Cancer—FRED C. COLLIER, M.D., J. F. A. MCMANUS, M.D., EDMUND A. DOWLING, M.D., and BEULAH M. HATHAWAY, M.D., University, Ala.; ROBERT H. KYLE, M.D., H. T. ENTERLINE, M.D., and STANLEY M. BECKER, M.D., Philadelphia, Pa.

11:00 a.m.–12:15 p.m.

6B Problems Related to Chronic Lung Diseases

Chemoprophylaxis in Pulmonary Emphysema—ANNE L. DAVIS, M.D., EVELYN J. GROBOW, M.D., JOHN H. MCCLEMENT, M.D., and RALPH R. TOMPSETT, M.D., New York, N. Y.

Kyphoscoliotic Cardiopulmonary Disease: A Reappraisal—GORDON L. SNIDER, M.D., GEORGE MILLER, BERNARD MILLER, and EDWARD I. ELISBERG, M.D., Chicago, Ill.

Effects of Kyphoscoliosis upon Mechanics of Breathing in Children and Adults—COLIN CARO, M.D., and THOMAS CUCKER, 3rd, M.D., Philadelphia, Pa.

The Place of Costo-Transversectomy in the Treatment of Scoliosis of the Thoracic Spine—LAMAR SOUTTER, M.D., Boston, Mass.; PAUL L. NORTON, M.D., Brookline, Mass.

Farmer's Lung: Clinical Physiology and Prognosis—QUINTON C. CALLIES, HELEN A. DICKIE, M.D., and JOHN RANKIN, M.D., Madison, Wis.

The Detection of Hepatic Toxicity Due to Pyrazinamide by Means of the Determination of Serum Enzymes—JOHN F. MORRISSEY, M.D., and RUTH C. RUBIN, B.A., Madison, Wis.

12:30 p.m.–1:45 p.m.

Sandwich Seminar IV*

The Current Status of Heart-Lung Devices Including the Supplemental Heart—CHARLES KIRBY, M.D., *Moderator*

Panelists: SELWIN MCCABE, M.D., Bethesda, Md.; DWIGHT MCGOON, M.D., Rochester, Minn.; JOHN C. SCHUDER, Ph.D., Philadelphia, Pa.

Sandwich Seminar V*

Esophageal Disease

Moderator and participants to be announced.

Sandwich Seminar VI*

Are Tuberculosis Case-Finding Programs in Need of Radical Revision?

Moderator and participants to be announced.

2:00 p.m.–3:15 p.m.

7A

Respiratory Disease in Europe, Africa, and Peru, 1957—HARRIET HARDY, M.D., Boston, Mass.

*Each Seminar will be limited to 30 physicians in order to allow for informal discussion with the experts. Tickets must be purchased in advance from the ATS. Locations for all of the Seminars to be announced.

The Effect of Viral Infections on Chronic Pulmonary Disease—FRED M. DAVENPORT, M.D., Ann Arbor, Mich.

3:30 p.m.–5:00 p.m.

7B Case Reports

1. JEROME E. COHN, M.D., CHARLES T. PINNEY, M.D., and TERENCE H. COCHRAN, M.D., Salt Lake City, Utah
2. GISELLA ALBRECHT, M.D., ADELAIDE DE ALONZO, M.D., WILLIAM B. BUCKINGHAM, M.D., and MABBU PARTHASARATHY, M.D., Chicago, Ill.
3. DAVID G. SIMPSON, M.D., New York, N. Y.
4. ELEANOR MALLAY SMITH, M.D., MAURICE SONES, M.D., and HAROLD L. ISRAEL, M.D., Philadelphia, Pa.
5. EDWARD A. GAENSLER, M.D., Boston, Mass.
6. EDWARD A. GAENSLER, M.D., Boston, Mass.

4:00 p.m.–5:00 p.m.

Demonstration V

Chest Conference, X-Ray Museum, Temple University—JACOB ZATUCHNI, M.D., *Moderator*

Limited to 50 physicians. Reservations must be secured in advance from the ATS.

Demonstration VI

Presentation of Patients with Sarcoidosis, Woman's Medical College, of Pennsylvania—MAURICE SONES, M.D., and HAROLD ISRAEL, M.D., *Moderators*

Limited to 50 Physicians. Reservations must be secured in advance from the ATS.

NOTICES

The following dates and locations of the next three annual meetings of the National Tuberculosis Association and the American Trudeau Society have been selected and approved:

1958—Philadelphia, Pennsylvania—May 18–23
1959—Chicago, Illinois—May 24–29
1960—Los Angeles, California—May 15-20

THIRD INTERNATIONAL CONGRESS OF ALLERGY

The Third International Congress of Allergy, sponsored by the International Association of Allergology and French Allergy Association, will be held in Paris, France, October 19–26, 1958.

All information regarding the program may be obtained from: Dr. Samuel M. Feinberg, 303 East Chicago Avenue, Chicago, Illinois, U. S. A.; Dr. José M. Quintero Fossas, Paceo 313, Vedado, Havana, Cuba; and Dr. Bernard N. Halpern, 197 Boulevard St. Germain, Paris 7, France.

ANNOUNCEMENT

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Whenever you are confronted with a patient who cannot tolerate PAS or who wants a more palatable form, you should find it of advantage to use BENZAPAS.

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[1]Dr. Samuel Phillips: *Calcium Benzoyl PAS*, Paper presented at 15th VA-Army-Navy Conference on Chemotherapy of Tuberculosis, St. Louis, Missouri, February 6-9, 1956.

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NATIONAL TUBERCULOSIS ASSOCIATION 1904–1954

A Study of the Voluntary Health Movement in the United States

by Richard Harrison Shyrock, Ph.D

W. H. WELCH PROFESSOR OF THE HISTORY OF MEDICINE, AND DIRECTOR OF THE INSTITUTE OF THE HISTORY OF MEDICINE, THE JOHNS HOPKINS UNIVERSITY.

342 pages, illustrated *$3.50*

This book would be a valuable addition to any medical library. It should be on the reference list of every medical school; and on the desk of all private physicians interested in the problems of tuberculosis.

Not only does it supply a historical background for the antituberculosis movement in the United States, but it affords enlightening orientation to the entire voluntary health movement with the National Tuberculosis Association as the principal illustration.

The three overlapping areas of the Association's activities—medical science and practice, public health, and social welfare—are presented here by an able medical historian who knows how to give facts in a clear and interesting way.

Among the chapters are: Medical Background; Social Background; Tuberculosis in the United States; 1904; Founding of the National Tuberculosis Association; Arousing the Public, 1904–1908; Growth of the Tuberculosis Movement, 1904–1920; Administrative Developments and Reorganization; Problems and Policies, 1908–1922; Widening Contacts and Activities, 1917–1922; Foreign Contacts, War, and the Emergence of a New Era; The Research Program, 1940–1954; Chemotherapy and the Climax of Fifty Years of Service.

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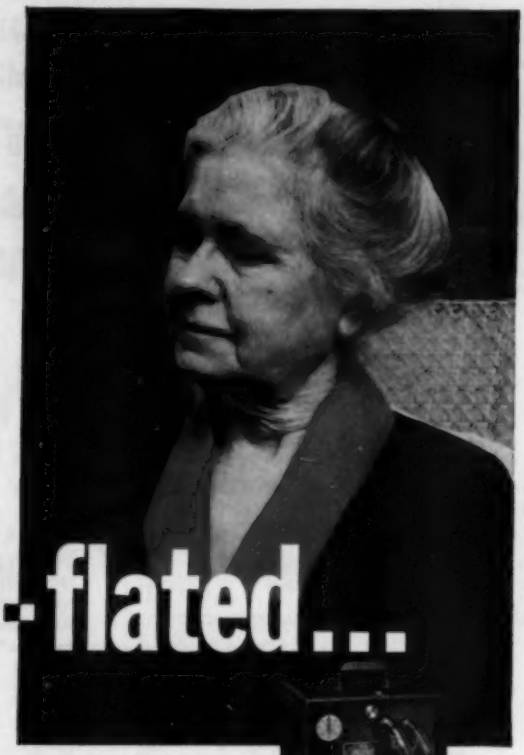

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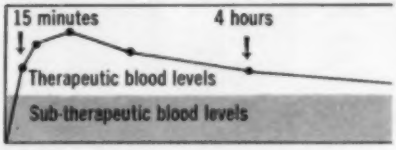

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